Chemical Analyses of Mosochiku (*Phyllostachys pubescens* Mazel) during Elongation Growth

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CONTENTS

General Introduction

Bamboos are perennial lignified plants and constitute Bambusoideae, a subfamily of Gramineae. Bamboos have a remarkable characteristic in developed vegetative growth organs with nodal branching and rhizome system, and widely distributes in the tropical, subtropical, temperate and sub frigid regions comprising about 700 species under 63 genera (Liese, 1987). According to rhizome systems bamboos are classified into three types : monopodial, sympodial and amphipodial types (Ueda, 1960). The bamboos commonly grown in the temperate region belong to the monopodial type, because their culms sprout out separately. On the other hand, the bamboos grown in the tropical region such as *bambusa* belong to the sympodial type because of their clumped culms. A group of bamboo grasses, *sasa* species, distributed mainly in the sub frigid region belongs to the amphipodial type because their rhizome systems have both characteristics.

In Japan, about 450 species are included in Bambusoideae, more than a half of them being classified as *sasa* species which mostly grow in Hokkaido district. The remaining species are exclusively grouped into the monopodial type and distributed in Honshu, Shikoku and Kyushu. Within monopodial type bamboos, Hachiku (*Phyllostachys nigra* Munro var. Henonis Makino), Madake (*Phyllostachys reticulate* C. Koch) and Mosochiku (*Phyllostachys pubescens* Mazel) are three big, popular and useful bamboos. The former two have been known as native species in Japan and used since ancient times, whereas Mosochiku was imported from China in *Edo* period, and afterward cultivated in wide area (Muroi, 1992). Now the cultivated area of Mosochiku is larger than the other two species.

From utilization point of view bamboo is a very important plant in Asia. First of all, bamboo is valuable for prevention against natural disasters and landscape protection. Stillness and beauty of bamboo planted at gardens casts a spell on our mind and appeases our fatigue with daily work. Vigorous growth of bamboo shoot from the mother land gives us a courage to live on hope. Bamboo has also been used as raw materials for house construction, household applications, weavings, handicrafts, paper, board, charcoal, and so on. The use of bamboo was extended to make musical instruments and elegantly designed arts and crafts such as tea-ceremony utensils and flower vases. Especially, bamboo in Kyoto stands high in the court tools on festivals as well as in tea ceremony and flower arrangement. In addition bamboo is known as one of important plants which constitutes a traditional combination of auspicious significance, *i.e.* pine, bamboo and Japanese plum branches (*sho-chiku-bai*). It is also noteworthy that bamboo is conducive to the culture of *Kanji* in Japanese writing. Furthermore, young bamboo shoot has been used as a food, *Takenoko* in Japan. Because of its delicacy, largeness and earliness of sprouting time, Mosochiku has been cultivated in wide area (36,254.5 ha in 1992 cited from the statistical table of Japanese Forest Agency 1993).

The most surprising feature of bamboo is rapidness of elongation growth. After sprouting out from soil, elongation growth of one culm of bamboo completes within a few months. The maximum heights of elongation per day in Mosochiku and Madake were recorded as 119 cm and 124 cm, respectively, at Nagaokakyo City, Kyoto, in 1954 (Ueda, 1960). This overall growth of bamboo is performed by summing up increase in length of elongation of constituting internodes, internodal growth. The author has been much impressed by this rapid elongation

rate of bamboo and fixed her eyes upon chemical features in relation to the growth of bamboo.

With respect of chemical properties of bamboo, lignification and related enzymes have been extensively studied by Higuchi and his coworkers (Higuchi, 1966; Higuchi *et al.* 1953, 1966, 1967a, b, 1969 Shimada *et al.* 1970), Itoh (1990), Yoshizawa *et al.* (1991) and Majima *et al.* (1991). Structure of hemicellulosic polysaccharides has been studied by Matsuzaki *et al.* (1960, 1962), Maekawa (1975a, b,), Maekawa and Kitao (1973, 1974), Yoshizawa and Idei (1981) and Kato *et al.* (1982). The gross chemical composition of bamboo shoot has been examined from the nutritional point of view (Koshimizu and Mitsui, 1955; Mori *et al.*, 1978; Kozukue *et al.*, 1981 and 1983; Mochizuki and Kurosaki, 1988). Since bamboo belongs to monocotyledon and its cell wall is known to contain appreciable amount of phenolic acids (Shimada *et al.*, 1971), several oligosaccharides esterified with ferulic and *p*-coumaric acids were isolated from enzymatic digest of bamboo shoot (Ishii *et al.*, 1990a, b, c, 1991). However, the relationship between elongation growth and chemical composition change was largely unknown. The results given in this field are limited to information about cellulose (Nomura and Yamada, 1974a; Idei, 1981) and L-tyrosine (Nomura and Yamada, 1974a, b). Only Shibamoto *et al.* (1954a, b) referred to change in chemical constituents of bamboo shoots after sprouting and gave results that chemical constituents of bamboo shoot harvested 20 days after sprouting resembled to those of mature bamboo, and that proteinic amino acid composition did not change accompanying its growth.

Recently, hemicellulose esterified with phenolic acid has been considered to play an important role in cell growth (Fry, 1979; Kamisaka *et al.*, 1990). Regarding to bamboo, although several

esterified oligosaccharides were isolated as described above, the origin of these oligosaccharides has not been clarified yet.

In addition, recent development in glycobiology suggests the importance of a glycoprotein, arabinogalactan protein (AGP), in growth of cabbage (Yasufuku *et al.*, 1994). Previous studies on AGP were exclusively carried out on dicotyledon (Clarke *et al.*, 1979; Fincher *et al.*, 1983) and no information was available in the monocotyledon including bamboo.

In this theses, the author focused on chemical composition change during elongation growth of Mosochiku by using its immature culm having 6 m in height.

The present theses constitutes following five chapters : in Chapter 1, chemical composition change of Mosochiku accompanying its whole growth was investigated (Fujii *et al.*, 1993a); in Chapter 2, composition change of Mosochiku accompanying its internodal growth was investigated (Fujii *et al.*, 1994a); in Chapter 3, histochemical approach was made to investigate distribution of phenolic acids in growing cell walls on immature Mosochiku culm (Fujii *et al.*, 1991, 1993b); in Chapter 4, a direct evidence of the presence of native partially acetylated arabinoxylan containing phenolic acids was presented (Fujii *et al.*, 1992, 1994b); and in the last Chapter 5, arabinogalactan protein as an indicator of immaturity of Mosochiku was investigated.

Chapter 1 Composition change of Mosochiku accompanying its whole growth

1.1 Introduction

Bamboo is an interesting plant because of its fast elongation growth and is a suitable material to investigate growth mechanism of higher plant. Previous chemical investigations on the growth of bamboo were mainly carried out on lignin and its biosynthesis (Higuchi, 1966; Higuchi *et al.*, 1953, 1966, 1967, 1969; Shimada *et al.*, 1970). As for other chemical constituents, Nomura and Yamada reported that the variation of L-tyrosine content could be followed by X-ray diffraction analysis (1974a) and that the crystallinity of the bamboo shoot covered with bamboo-sheath increased from the top to the bottom (1974b). Idei (1981) reported that degree of polymerization of cellulose increased with growth and reached to the same value as that of mature bamboo at 2 months after sprouting out. Taniguchi (1956) also reported that crystalline region of bamboo shoot became larger with its growth. Shibamoto *et al.* analyzed the variation of chemical constituents of bamboo shoots harvested every few days after sprouting and showed that the constituents of bamboo shoot given at 20 days after sprouting were essentially the same as those of mature bamboo culm (1954a). They also reported that proteinic amino acid composition did not change accompanying its growth (1954b). These results indicate that an immature bamboo culm is a useful material to investigate the chemical composition changes accompanying its growth.

In this chapter it is described that a detailed chemical composition change from the top to the bottom of an immature Mosochiku culm having about 6 m in height.

1.2 Materials and methods

1.2.1 Plant materials

An immature culm and an edible shoot of Mosochiku were harvested on May 13, 1989, at the Botanical Garden of Kyoto University. The heights of the culm and shoot were 633 cm and 38 cm, respectively. The immature bamboo culm was enveloped with bamboo-sheaths up to 2 m from the bottom, while the whole edible bamboo shoot was enveloped with bamboo-sheaths. Immediately after harvesting, the immature bamboo culm was cut into 6 portions 100 cm in length, except for the top portion which was 133 cm in length, following which their sheaths were peeled off. The divided 6 portions of the immature bamboo culm, designated as No.1 to No.6 from the bottom to the top of the culm as shown in Fig. 1.1, were cut into small pieces, lyophilized and milled to pass 24-mesh screen by a Wiley mill. The edible bamboo shoot was treated similarly and designated as EBS.

1.2.2 Chemical composition analyses

The contents of moisture, ash, alcohol-benzene extract, cold water extract, 1 % sodium hydroxide extract and pentosan were respectively determined by the Standard Methods (P-8002, 8003, 8010, 8004, 8006 and 8011) of the Japanese Industrial Standard (JIS). The Klason and acid-soluble lignin were respectively determined by the Standard Methods (T222-os-74 and UM250) of the Technical Association of Pulp and Paper Industry (TAPPI). Uronic acid anhydride was determined by the method of Johansson *et al.* (1954). Acetyl was determined by gas liquid chromatography (GLC) on a column of DB-Wax (0.25 mm x 30 m) at 120 °C after hydrolysis of the dried sample with 1 N hydrochloric acid at 100 °C for 2 hr with 1-propionic acid being used as an internal standard. GLC was carried out by using a Shimadzu GC-15A gas

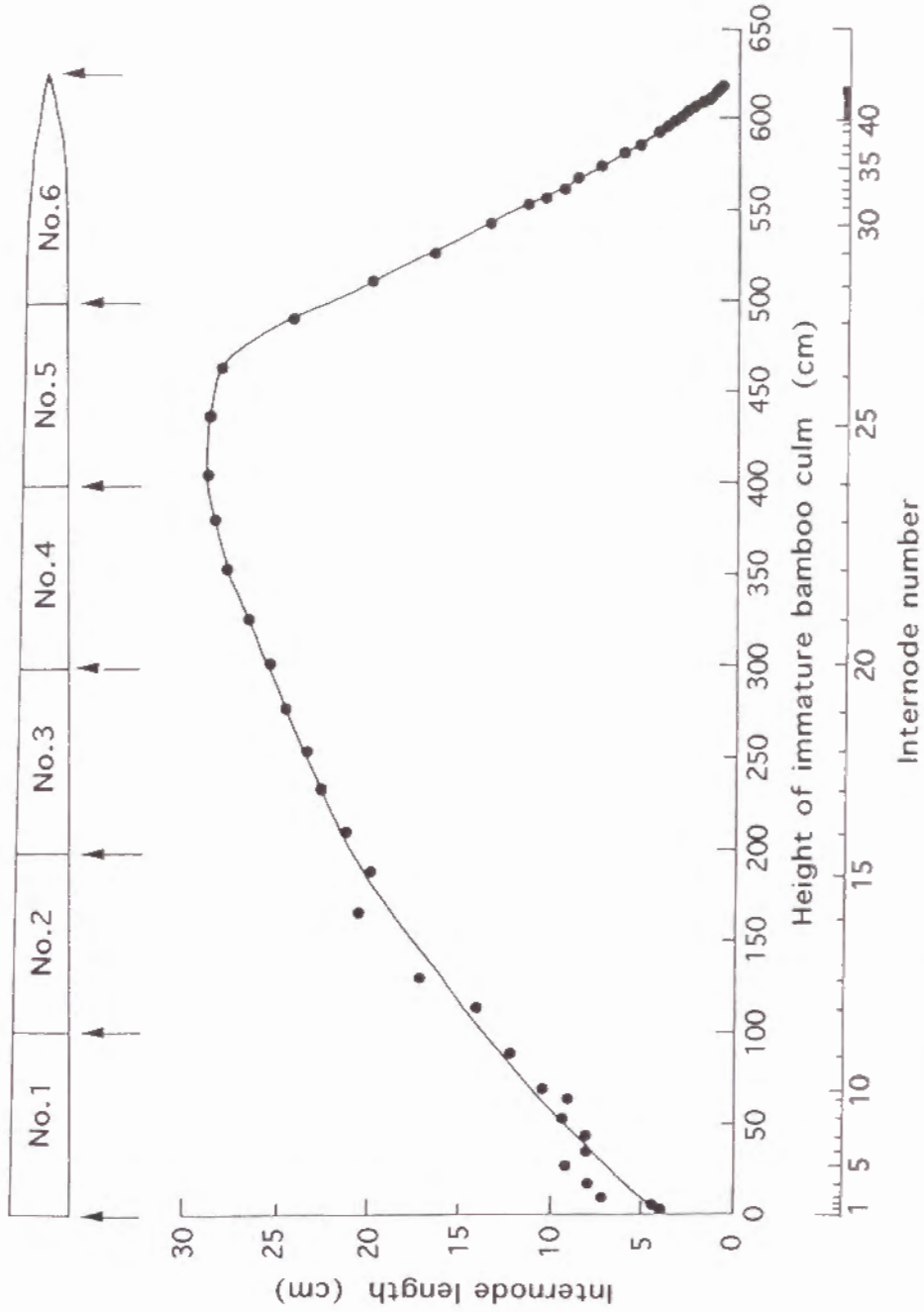

Fig. 1.1 Relation of internode length with its serial numbers and height of immature Mosochiku culm, and positions of samples. An immature culm of bamboo 633 cm in height was cut into 6 portions (No.1-6) at positions indicated by arrows.

chromatograph equipped with flame ionization detectors. The holocellulose and α-cellulose contents were determined by the method of Uprichard (1965). The starch content was determined by the method of Humphreys and Kelly (1961). The nitrogen content was determined by the method of Kjeldahl, and the protein content was estimated by multiplying the nitrogen content by factor of 6.25.

1.2.3 Crystallinity indices of cellulose

Dried native bamboo meal was compressed into a disc (20 mmϕ x 1 mm) and subjected to X-ray diffractometry by a Rigaku Geigerflex 2011B equipped with a line focusing X-ray tube and Ni-filtered CuKα (35 kV, 20 mA) radiation. Crystallinity indices of cellulose were calculated according to the method of Segal *et al.* (1959).

1.2.4 Neutral sugar composition

An extractive-free sample was hydrolyzed with 72 % sulfuric acid followed by dilution to 4 % sulfuric acid according to Saeman *et al.* (1954), and the neutral monosaccharides were converted into alditol acetates and analyzed by GLC on a column of DB-225 (0.25 mm x 15 m) at 220 °C.

1.2.5 Phenolic acid and phenolic aldehyde composition

Dried native sample was saponified in the dark with 2 N sodium hydroxide solution for 24 hr at room temperature under nitrogen gas. After filtration, the filtrate was adjusted to pH 2.1 with hydrochloric acid, and extracted three times with hexane to remove free fatty acids (Dabrowski and Sosulski, 1984). Phenolic acids and phenolic aldehydes were then extracted 5 times with dichloromethane. The dichloromethane solution was evaporated to dryness, trimethylsililated with TMS-BA at 40 °C for 15 min, and analyzed by GLC on a column of CBP-1 (0.25 mm x 25 m) at 200 °C using methyl heptadecanoate as an internal standard.

1.2.6 Amino acid analysis

Free amino acids were extracted 5 times from dried sample with 75 % aq. methanol for 24 hr at room temperature. An aliquot of the solution was diluted with 0.2 N citrate buffer (pH 2.2), and the free amino acid was analyzed by a Hitachi 835 amino acid analyzer equipped with a Na-type #2619F resin column (4 x 150 mm).

1.2.7 CP/MAS ^{13}C-NMR spectroscopic analysis

CP/MAS ^{13}C-NMR measurements were performed on a Chemagnetics CMX300 spectrometer operating at 7.04 T which corresponded to a frequency of 75.3 MHz for ^{13}C. Magic angle spinning was carried out at a frequency of 4-6 KHz with a Chemagnetics Omnimatch Magic Angle Rotor Module and a Zirconia 7.5 mm (o.d.) bullet-type rotor. Contact time and repetition time were settled to 1 ms and 3.0 s, respectively, for usual CP/MAS experiments. The proton spin-lattice relaxation time constant in rotating frame, $T_1\rho$(H), was determined by measuring signal strengths over a range of proton spin-locking periods prior to the contact time (Schaefer *et al.*, 1977). Chemical shifts were referred to tetramethylsilane by using hexamethylbenzene as a secondary reference (methyl peak at 17.4 ppm).

1.3 Results and discussion

1.3.1 Increase and decrease in internode length

Figure 1.1 shows relation of internode length with its height, beginning from the lowest to the top of the culm of Mosochiku used in this study. The pattern of internode length showed a peak at the 24th internode (about 4 m in height). The elongation rate of the internodes

lower than this peak (samples No.1-4) seems to be diminishing with decrease in height. The internode No.1 has completely stopped the elongation by loss of sheath which was indispensable for growth. In contrast, elongation of the internodes higher than the peak (samples No.5 and 6) is in progress. The pattern shown in Fig. 1.1, which is similar to the results by McClure (1966), and Nomura and Yamada (1974a, b), indicates that the present samples are appropriate for the study of chemical composition change accompanied by the growth.

1.3.2 General chemical compositions

Table 1.1 summarizes chemical compositions of the 6 portions (No.1-No.6) of the same culm and the edible bamboo shoot (EBS). The moisture content increased with height, and the samples No.5 and No.6 gave a similar value (90.3%) to that of EBS (91.7%). More than 80% of the dry weight of the culm (81.5%, w/w) was in the lower half (No.1-3), indicating that contribution of the upper portion to the chemical composition was mostly cancelled if the whole culm was analyzed.

From the results listed in Table 1.1, the contents of three major constituents, cellulose, hemicellulose and lignin, became lower of higher portions. The uronic acid and acetyl contents changed parallel to these major constituents, suggesting that these components were linked to hemicelluloses, or linked each other to form pectin in the case of uronic acid. However the protein and mineral (ash) contents increased with increase in height. The inverse relationship of acid-soluble lignin and the Klason lignin content may be due to the higher protein content in higher positions, since an UV spectroscopy was used for estimation of acid-soluble lignin. The upper portions such as No.5 and No.6 contained much more water-soluble and alkali-soluble components than in the lower portions such as No.1 and No.2, and the chemical composition of the top portion, No.6, is similar to that of EBS.

Table 1.1 Summative chemical compositions and crystallinity indices of Mosochiku bamboo culm and its edible bamboo shoot (weight %)

Component	Positions of immature bamboo culm						
	No.1	No.2	No.3	No.4	No.5	No.6	EBS
Moisture content	79.4	80.0	84.6	88.5	90.3	90.3	91.7
Dried weight matter	40.3	25.6	15.6	9.2	6.4	2.9	
	(978)[a]	(620)	(378)	(224)	(155)	(70)	(152)
Alcohol-benzene extract content	9.2	6.9	11.8	15.0	18.0	11.6	15.5
1% NaOH extract content	40.9	40.0	47.2	58.6	71.1	86.5	72.5
Cold water extract content	15.3	16.7	25.2	38.9	46.9	48.7	57.5
Lignin content							
Klason	8.8	8.2	5.4	2.6	0.6	0.3	0.4
Acid-soluble	3.0	3.6	4.1	6.3	9.1	11.7	12.2
Holocellulose content	80.6	81.1	71.0	65.2	53.1	39.8	39.9
α-Cellulose content	47.3	48.8	37.6	32.6	23.0	13.9	12.0
Pentosan content	24.5	34.3	28.0	21.9	11.4	5.1	7.2
Uronic acid anhydride content	4.3	4.4	4.5	3.4	3.4	3.4	2.6
Acetyl content	4.0	4.8	4.0	3.5	1.6	0.6	0.8
Starch content	1.2	1.4	2.0	3.8	6.1	7.3	7.8
Protein content	5.0	5.8	7.6	11.7	19.6	30.6	28.0
Ash content	2.0	2.1	3.1	5.0	7.5	10.8	8.0
Phenolic acid content	0.521	0.514	0.352	0.356	0.124	0.039	0.035
Phenolic aldehyde content	0.028	0.014	0.010	0.004	0.002	0.001	0.001
Crystallinity index	44.1	43.2	40.7	32.2	23.2	8.5	5.8

[a] Dried weight of materials actually obtained (gr).

Bamboo has been known to contain ferulic and *p*-coumaric acids both in free (Shimada *et al.*, 1970) and bound (Shimada *et al.*, 1971, Azuma *et al.*, 1985; Ishii *et al.*, 1990a, b, c) states. Preliminary examination indicated the presence of phenolic aldehydes. Accordingly phenolic acid and phenolic aldehyde within the same culm and the edible bamboo shoot were analyzed. As shown in Table 1.1, phenolic acids increased from the top to the bottom in relation to the lignin content which is consistent with the reported finding (Shimada *et al.*, 1970). This study further showed that distribution of phenolic aldehydes in the bamboo was similar to that of lignin.

Based on the chemical composition data, it can be concluded that the immature bamboo culm used in this chapter is vertically constituted of portions with different maturity.

1.3.3 Neutral carbohydrate composition

As to the neutral carbohydrate moiety, the upper portions (No.5 and No.6) and the edible bamboo shoot were rich in glucose residues, and the other sugars, except xylose, were also rich in the upper portions as shown in Table 1.2. From the α-cellulose and starch contents

Table 1.2 Neutral sugar composition of immature Mosochiku culm and its edible bamboo shoot

(relative weight %)

Component	Positions of immature bamboo culm						EBS
	No.1	No.2	No.3	No.4	No.5	No.6	
L-Rhamnose	0.3	0.4	0.6	0.5	0.6	0.9	0.7
L-Arabinose	2.0	2.0	3.1	2.8	4.4	7.6	6.4
D-Xylose	37.5	38.2	34.9	30.6	21.2	13.3	9.9
D-Mannose	0.3	0.4	0.9	1.0	1.1	2.3	1.2
D-Galactose	0.8	0.9	1.9	1.9	3.2	7.0	5.1
D-Glucose	59.0	58.1	58.8	63.1	69.6	69.0	76.7

(Table 1.1), the glucose residue in the upper portions (No.5, No.6) was mainly ascribed to starch, while that in the lower portions was to cellulose. Some glucose, present in the edible bamboo shoot, may be derived from xyloglucan and (1,3;1,4)-β-D-glucan characterized by Kato *et al.* (1982).

A remarkable feature in Table 1.2 is the steady increase in xylose content from the top to the bottom. The xylan content reaches a plateau at section No.2, almost in parallel with the Klason lignin content. This strongly suggests a simultaneous biosynthesis of lignin-hemicellulose network.

1.3.4 Phenolic acid and phenolic aldehyde composition

Table 1.3 shows phenolic acid and phenolic aldehyde contents within one culm and of the edible bamboo shoot. Five phenolic acids and 3 phenolic aldehydes were found in the lower portions (No.1, No.2), while syringaldehyde and syringic acid were not detected in the top portion, No.6 and the edible bamboo shoot. About 90% of phenolic acid was composed of *p*-coumaric acid and ferulic acid, both being exclusively in *trans* form. If samples were exposed to sunlight for a long time, a substantial amount of these acids was converted into *cis* form. The content of *p*-coumaric acid increased from the top to the bottom in accordance with the lignin content as reported by Shimada *et al.* (1970), while a reverse relationship was observed in the ferulic acid content. The ratio of ferulic acid / *p*-coumaric acid fell to smaller than unity at the position No.2. Recently it has been suggested that ferulic acid plays an important role in regulation of elongation growth (Fry, 1979, 1983; Kamisaka *et al.*, 1990). The present results support this suggestion but further work is necessary to clarify the exact role of ferulic acid in the bamboo cell wall.

Table 1.3 Phenolic acid and aldehyde composition of immature Mosochiku culm and its edible bamboo shoot

(relative weight %)

Component	Positions of immature bamboo culm						
	No.1	No.2	No.3	No.4	No.5	No.6	EBS
p-Hydroxybenzaldehyde	3.0	1.7	1.2	0.4	1.4	1.9	1.6
p-Hydroxybenzoic acid	0.4	0.2	0.2	0.6	3.0	6.5	3.7
Vanillin	1.3	0.7	0.6	0.5	0.5	0.8	1.3
Vanillic acid	0.1	0.1	0.3	0.4	0.1	2.9	4.2
Syringaldehyde	0.7	0.3	0.2	0.1	0.2	-	-
Syringic acid	0.1	0.1	-	-	-	-	-
trans-*p*-Coumaric acid	54.8	48.6	45.5	29.0	24.9	15.9	11.5
trans-Ferulic acid	39.5	48.2	51.9	68.9	68.8	72.1	77.7

1.3.5 Crystallinity

Figure 1.2 shows a variation of X-ray diffractometry within one immature bamboo culm and edible bamboo shoot. Table 1.1 also lists crystallinity indices evaluated according to the method of Segal *et al.* (1959). The crystallinity indices increased with lowering the position of the culm in accordance with the increase of α-cellulose. Cellulose is essentially the sole crystalline material in wood, but bamboo contains a large amount of free tyrosine as shown in Table 1.4 and as also reported by Nomura and Yamada (1974a), Shimada *et al.* (1970) and Kozukue *et al.* (1983), some of the tyrosine in bamboo was shown to be crystalline. In fact the crystallinity indices shown in Table 1.1 seem to be too high in comparison with estimated value from the cellulose content. The X-ray diffractograms shown in Fig. 1.2 are overlapped patterns of amorphous hemicellulosic polysaccharides, starch, tyrosine and cellulose. The present results should be interpreted as showing that crystalline cellulose content rapidly increased up to No.3, and then gradually increased.

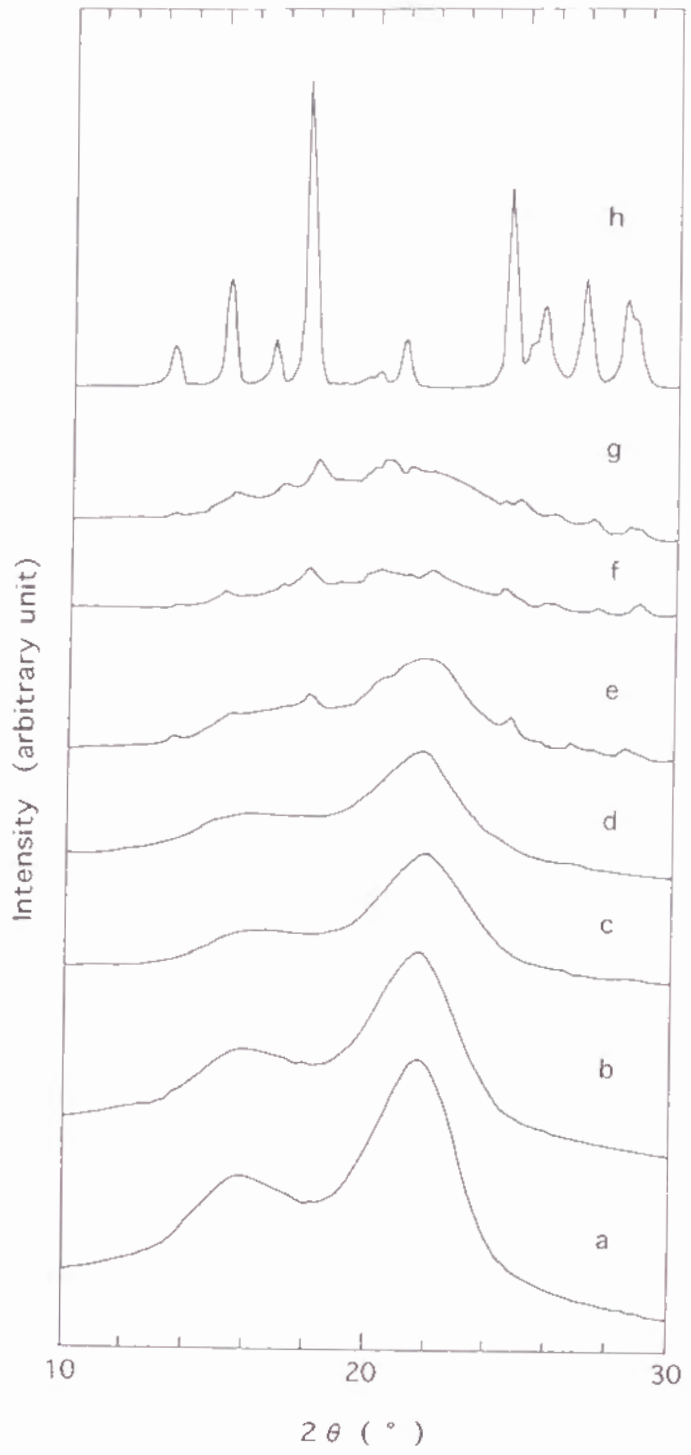

Fig. 1.2 X-ray diffraction patterns of six different portions of immature Mosochiku culm, its edible bamboo shoot and L-tyrosine.
(a), No.1; (b), No.2; (c), No.3; (d), No.4; (e), No.5; (f), No.6; (g), EBS; (h), L-tyrosine.

1.3.6 Free amino acid composition

Table 1.4 summarizes free amino acid composition within one immature culm of Mosochiku. Sixteen free amino acids were detected in the bamboo culm. Taurin and *O*-phosphoserine were also detected, but could not be quantitatively estimated because of low concentration. The present results on the edible bamboo shoot confirmed the previous finding that L-tyrosine was the predominant amino acid (Nomura and Yamada, 1974a). As for the immature bamboo culm, the L-tyrosine content was highest at the top, decreased between No.6 and No.4, and rapidly decreased between No.4 and No.2. Since L-tyrosine is a

Table 1.4 Free amino acid composition of immature Mosochiku culm and its edible bamboo shoot

(mg / dried sample 100g)

Component	Positions of immature bamboo culm						EBS
	No.1	No.2	No.3	No.4	No.5	No.6	
Ala	4.6	3.2	5.3	12.2	18.6	88.2	45.0
Arg	4.6	3.9	4.0	4.2	4.3	15.4	15.9
Asp	9.8	2.9	4.2	19.4	30.4	66.6	66.8
Cys	3.2	2.5	3.7	4.3	4.6	9.0	7.6
Glu	3.7	7.9	7.8	10.2	13.0	60.7	21.3
Gly	1.9	1.2	1.8	1.3	1.6	4.8	2.9
His	-	-	1.1	1.8	1.9	3.4	5.1
Lys	3.4	1.3	5.3	3.9	3.8	7.0	5.6
Met	5.1	3.3	6.4	6.1	6.8	-	5.2
Phe	4.0	2.0	5.1	6.4	8.4	20.3	32.2
Pro	7.8	3.7	10.3	13.0	22.2	50.1	47.5
Ser	4.2	1.6	2.7	3.8	4.9	17.0	6.7
Thr	3.7	1.4	8.3	19.3	19.6	9.5	6.4
Tyr	23.3	10.7	182.9	636.7	625.7	1554.8	1818.0
Val	8.2	4.3	9.5	11.7	15.5	46.2	40.5
γ-ABA	6.8	4.3	5.0	4.9	5.4	21.3	16.2
Total	94.3	54.2	263.4	758.2	786.7	1974.3	2143.0

precursor of lignin, lignin biosynthesis could be progressed at the middle portion of the immature bamboo culm (No.3, No.4). This indicates that lignification was not advanced at this stage of growth. In fact, Higuchi *et al.* (1953) reported that parenchyma tissue, other than vascular bundle, was negative to phloroglucinol and Mäule reactions until a Mosochiku shoot grew to about 5 m in height. Recently, Itoh (1990) also reported that parenchyma cells in the outside of the 2nd internode of a Madake culm 580 cm in height initiated the lignification.

1.3.7 CP/MAS ^{13}C-NMR spectroscopic analysis

Figure 1.3 shows height dependency of solid state ^{13}C-NMR spectra for one immature bamboo culm and edible bamboo shoot. The spectra of the lower portions (No.1 to No.3) were similar to those of lignocelluloses with low lignin content (Kolodziejski *et al.* 1982). With ascending height, eight signals at 37, 58, 113, 115, 120, 133, 154 and 177 ppm became evident and sharper, and the strongest in the edible bamboo shoot (Fig. 1.3g). By comparing the spectrum of crystalline L-tyrosine as shown by asterisks in Fig. 1.3h, these signals were assigned to L-tyrosine. The spectrum of L-tyrosine shown in Fig. 1.3h was contaminated with several spinning side bands, but was completely coincident with that shown by Fyfe (1983).

In addition, the edible bamboo shoot was extracted five times with 75 % aq. methanol, and the solid state ^{13}C-NMR spectra of the extract and the extracted residue were compared with that of native edible bamboo shoot as shown in Fig. 1.4. The spectrum of the aqueous methanol extract accumulated L-tyrosine together with the other lower molecular weight materials. In turn, the extracted residue was completely freed from signals due to L-tyrosine. Furthermore, the values of $T_1\rho$(H) of L-tyrosine (2.5-2.7 msec) did not change compared to those of standard crystalline L-tyrosine. Although the results of

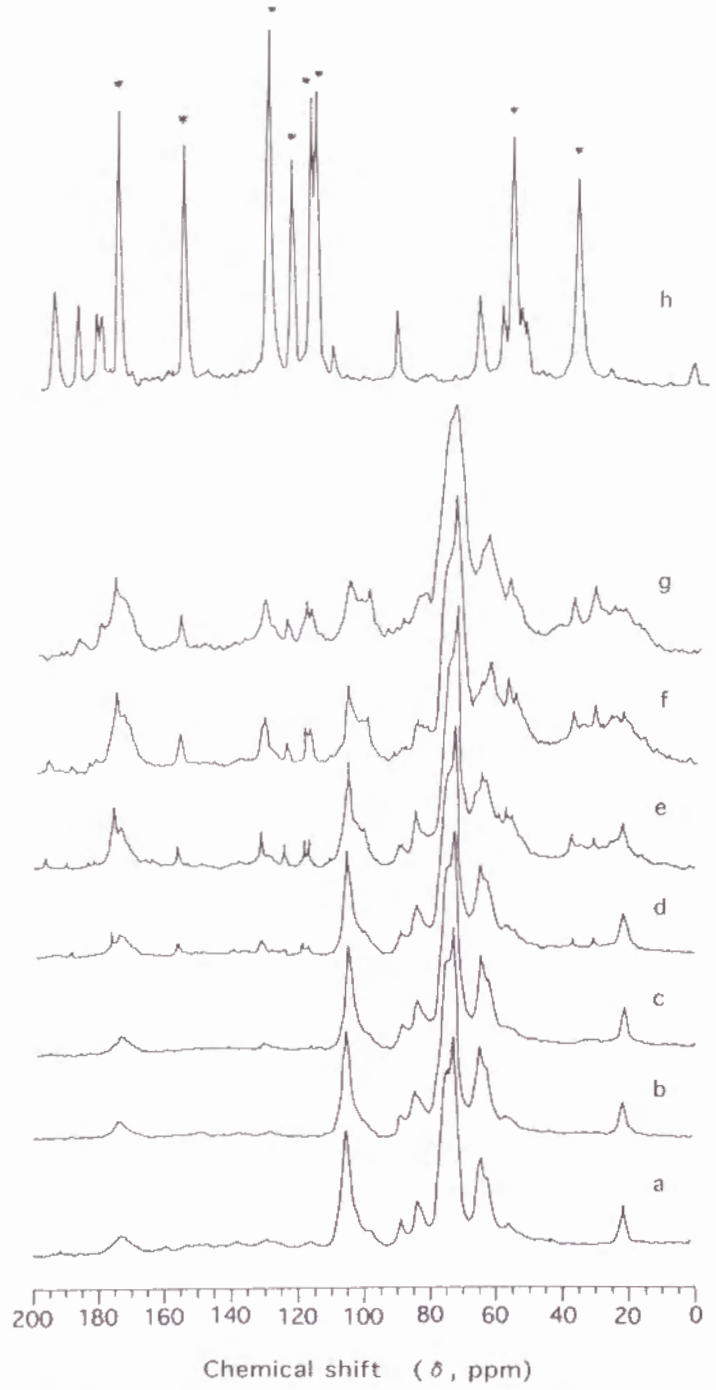

Fig. 1.3 CP/MAS ^{13}C-NMR spectra of six different portions of immature Mosochiku culm, its edible bamboo shoot and L-tyrosine.
(a), No.1; (b), No.2; (c), No.3; (d), No.4; (e), No.5;
(f), No.6; (g), EBS; (h), L-tyrosine.
The signals shown by asterisks in (h) were due to L-tyrosine and all the other weak signals were of spinning side bands.

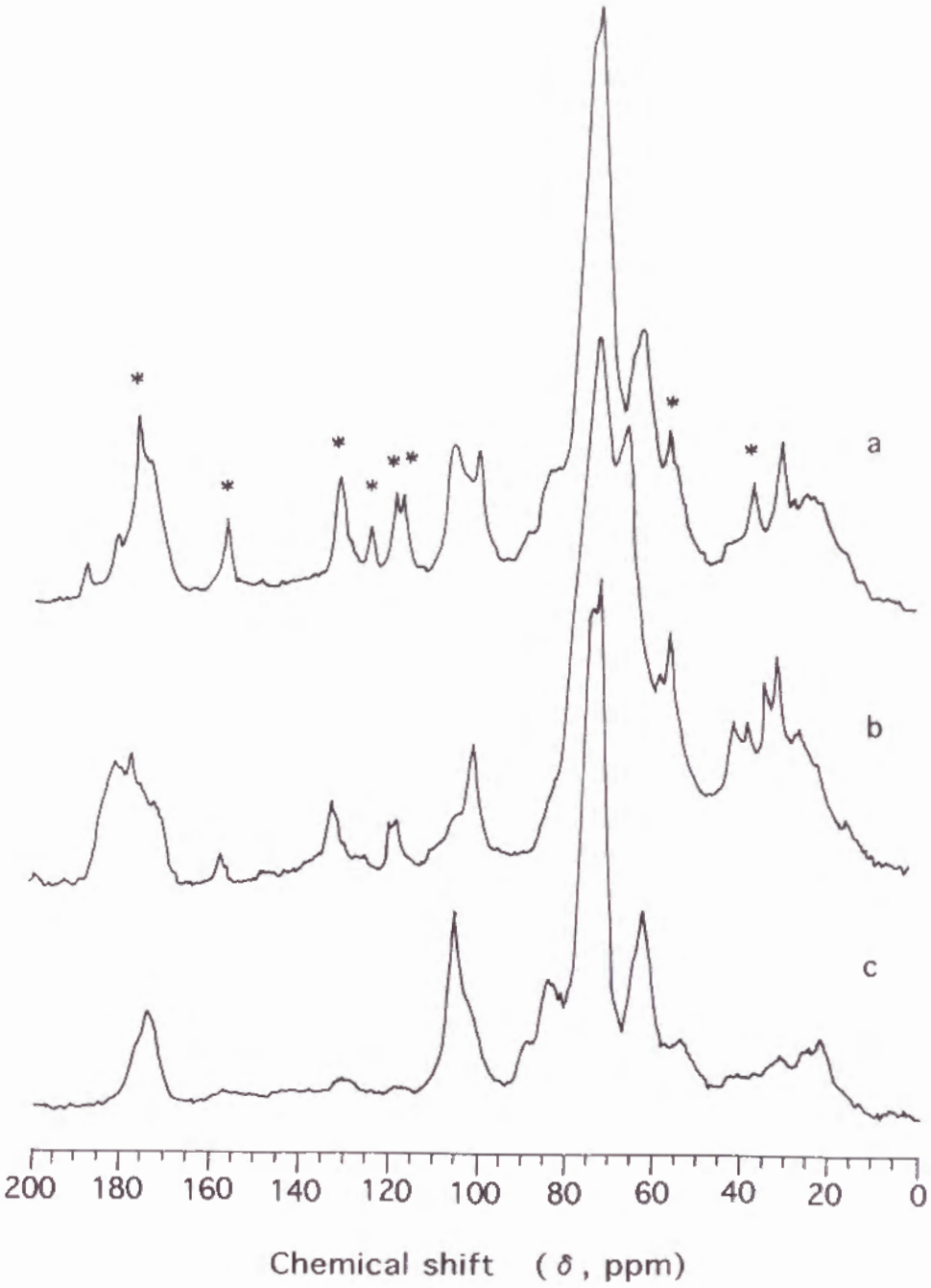

Fig. 1.4 Effect of 75% aqueous methanol extraction on CP/MAS ^{13}C-NMR spectra of edible bamboo shoot.
(a), native edible bamboo shoot;
(b), 75% aqueous methanol extract;
(c), residue obtained after 75% aqueous methanol extraction.
The signals shown by asterisks in (a) were due to L-tyrosine.

$T_1\rho$(H) were difficult to interpret by spin-diffusion processes, the results indicated that L-tyrosine was present as widely dispersed crystals in the freeze-dried tissues of edible bamboo shoot and not formed a complex with the other components. Previously, Nomura and Yamada (1974a) showed the presence of crystalline L-tyrosine in an immature bamboo culm by X-ray diffraction analysis. Present results further demonstrated the presence of tyrosine by solid-state ^{13}C-NMR spectroscopy. Since sensitivity of solid-state ^{13}C-NMR spectroscopy was appreciably higher than that of X-ray analysis as evidenced from the results in Figs. 1.2 and 1.3, solid state NMR is a powerful technique for analysis of growth of bamboo.

From the carbohydrate polymer perspective, Figure 1.4c is the key spectrum. The prominent shoulder attached to the main cellulose peak at 106 ppm has been shown to be due to the presence of xylan in Esparto grass (Marchessault *et al.*, 1985). The general assignment of the peaks in Fig. 1.4c to cellulose and xylan carbons and groups is now well established (Taylor *et al.*, 1983).

1.4 Summary

An immature culm of Mosochiku 6 m in height, was divided by 1 m each into 6 portions and their chemical compositions were analyzed using an edible bamboo shoot as a reference. The results indicated that the contents of cellulose, hemicellulose and lignin in the immature bamboo culm became higher with lowering the position of the culm. The increase of cellulose in the lower position was accompanied by increase of crystallinity. Uronic acid and acetyl group changed along with these constituents. The contents of phenolic acid and phenolic aldehyde were also higher in the lower portions, whereas the protein, starch and ash contents were higher in the higher portions. About 90 % of phenolic acid was composed of *p*-coumaric acid and ferulic acid both being exclusively in the *trans* forms, but their distribution profile was different: *p*-coumaric acid increased from the top to the bottom of the culm in relation to the lignin content, while a reverse relationship was observed in the ferulic acid. Sixteen free amino acids were detected of which L-tyrosine was predominant. The content of L-tyrosine was the highest at the top, and decreased rapidly at the middle portion, 3-4 m in height of the immature bamboo culm where lignin biosynthesis rapidly progressed. CP/MAS ^{13}C-NMR spectroscopy was found to be useful to analyze distribution of L-tyrosine. The present results showed that the immature Mosochiku culm having 5-6 m in height was appropriate to investigate the growth of Mosochiku.

Chapter 2 Composition change of Mosochiku accompanying its internodal growth

2.1 Introduction

In the previous chapter it was shown that the immature Mosochiku culm having 6 m in height was appropriate to investigate its whole growth by analyzing height-dependent chemical composition change.

The overall growth of bamboo is performed by summing up increase in length during elongation of constituting internodes (internodal growth). Although the internodal growth has been known to occur in zones of intercalary meristem each located close to a lower end (McClure, 1966), exact position has not been elucidated. Shibamoto *et al.* (1954a) and Nomura and Yamada (1974a, b) divided an internode of Mosochiku into three portions and examined chemical composition and X-ray diffraction pattern of each portion, respectively. Their results showed that the lower portion was younger than the upper one. Itoh (1990) analyzed longitudinal progress of lignification in the culm of Madake within one growing season by staining thin sections with Wiesner's reagent and UV-microscopy, and reported that lignification within one internode proceeded downward from the upper to the lower side.

These previous results suggest the presence of a height dependent gradation of chemical compositions within one internode reflecting differences in growth stage. However, no such investigation has been available. Therefore in this chapter, the chemical composition change accompanying internodal growth was characterized by using the longest internode of growing 6 m-high Mosochiku, and the exact position of the intercalary meristem was determined.

2.1 Materials and methods

2.1.1 Plant materials and general methods

An immature 594 cm-high Mosochiku culm was harvested on May 14th, 1992, at the Botanical Garden of Kyoto University. The relationship between culm height and internode length is shown in Fig. 2.1. This profile is similar to the data shown in Fig. 1.1. The internode length attained maximum at 17th internode. At the portion lower than 15th internode, the elongation growth has already finished because of loss of bamboo-sheath, but the portions upper than this internode would continue elongation as suggested by Nomura and Yamada (1974a, b). At upper internodes close to the 15th internode the elongation growth will approach to completion and lignification will be initiated. Therefore, the longest 17th internode, 342.7-372.5 cm from the ground (29.8 cm in length) was taken as a sample. The 17th internode was cut into 28 portions, 1 cm in height, by a band-saw (AEW Engineering Co., Ltd, Type 350 Series R, UK) equipped with a blade having thickness of 0.35 mm and width of 15.8 mm. The portions were numbered as No.1 to No.28 from the bottom (Fig. 2.2). Sixteen positions including fourteen even numbered portions and two odd numbered bottom portions (No.1 and 3) were lyophilized and milled as described in 1.2.1.

Unless otherwise specified materials, methods and instruments were the same as those described in Chapter 1.

2.2.2 Chemical analyses

Total sugar content was measured by the phenol-sulfuric acid method (Dubois *et al.*, 1956) after Saeman's hydrolysis method (Saeman *et al.*, 1954).

Phenolic acid contents as ferulic and *p*-coumaric acids were determined as three types, *i.e.* free, soluble-bound and insoluble-bound

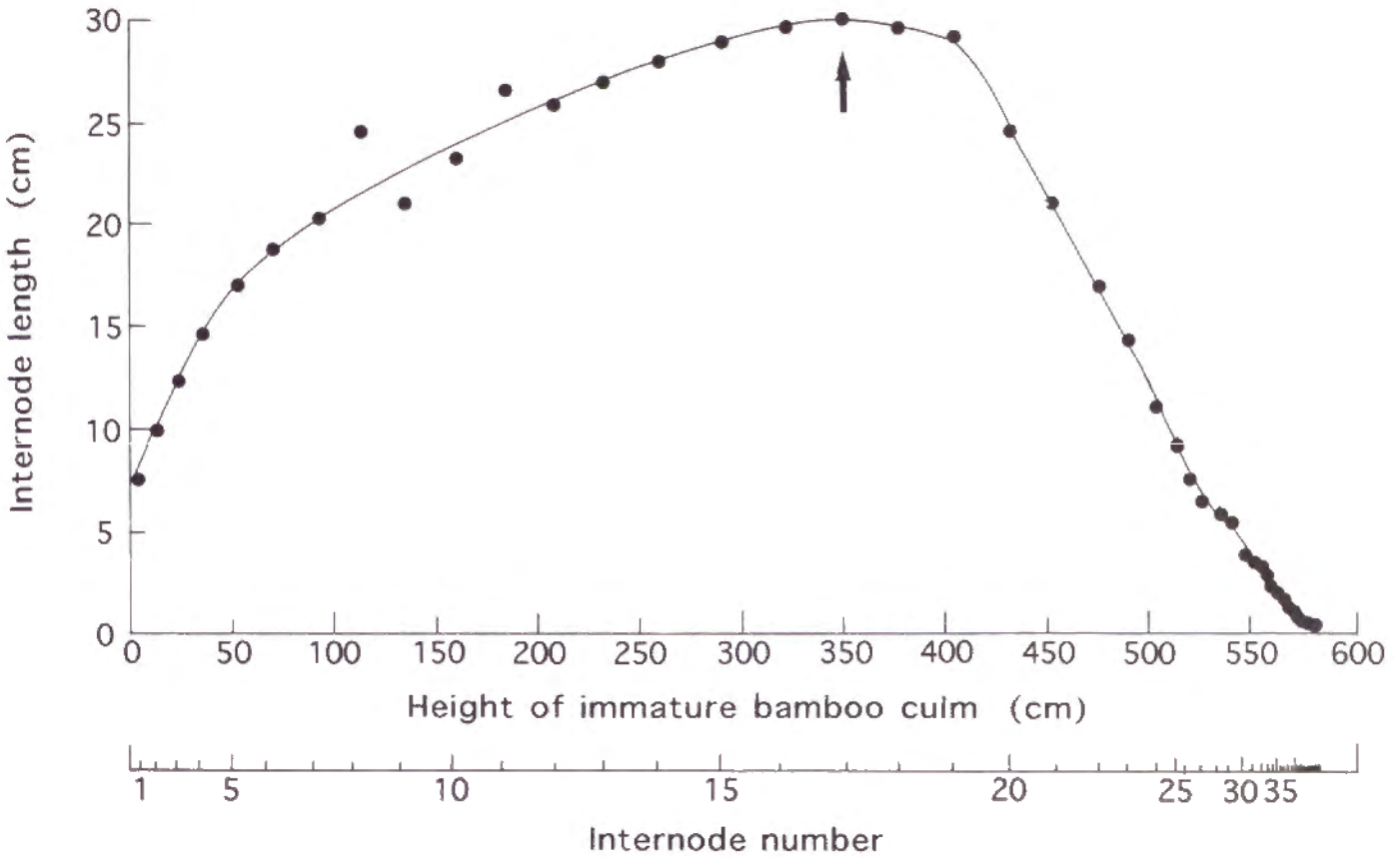

Fig. 2.1 Relation of internode length with its serial numbers and height of immature Mosochiku culm. An arrow shows the longest 17th internode of immature culm of bamboo 594 cm in height.

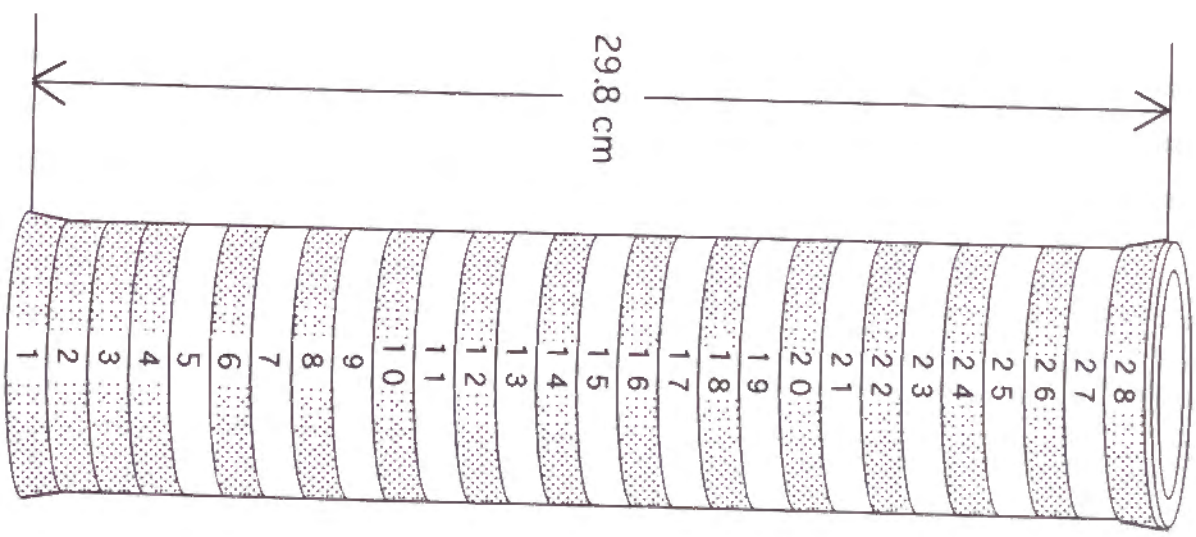

Fig. 2.2 Schematic illustration of samples taken from 17th internode (29.8 cm in height), each having 1 cm in height.

types (Krygier *et al.*, 1982). Dried sample was extracted 5 times with a 1:1 (v/v) mixture of 70 % aq. methanol : 70 % aq. acetone. The extract was adjusted to pH 2.1 with hydrochloric acid and washed 3 times with hexane to remove fatty acid. The aqueous phase was extracted with ethyl ether to recover free phenolic acids. The remaining insoluble residue and aqueous phase were saponified with 2 N sodium hydroxide for 24 hr to release insoluble and soluble-bound phenolic acids, respectively, which were recovered as described above. The phenolic acids contents were analyzed as TMS-derivatives by GLC as described in 1.2.5.

2.2.3 X-ray diffractometric analysis

X-ray diffractions were performed on a Rigaku RINT 1200 diffractometer (40 kV and 30 mA) equipped with a reflection type goniometer, using Ni-filtered CuKα radiation, and the crystallinity indices of cellulose portions were estimated according to Segal *et al.* (1959).

2.3 Results and discussion

2.3.1 General chemical compositions

The change of chemical compositions within the longest internode (No.17) along the longitudinal direction is shown in Table 2.1. The most remarkable characteristic was noticed in the sliced sample No.2 which covered from 1.06 to 2.13 cm from the lower end node. This sample had the lowest Klason lignin, holocellulose and α-cellulose contents, but showed the highest values in the acid-soluble lignin, starch and protein contents. These values gradually changed with ascending height in the direction where deposit of cellulose, hemicellulose and lignin progressed. Longitudinal change of crystalline indices, measured by X-ray diffraction as shown in Fig. 2.3, supported this upward

Table 2.1 Summative chemical compositions of 17th internode of immature Mosochiku culm
(weight %)

Component	Sliced sample number							
	No.1	No.2	No.3	No.4	No.6	No.8	No.10	No.12
Dried weight matter	3.8 (2.64)[a]	3.0 (2.09)	3.1 (2.12)	3.1 (2.15)	3.0 (2.06)	3.0 (2.06)	3.2 (2.18)	3.4 (2.33)
Lignin content								
Klason	1.7	0.4	0.6	1.0	2.0	1.7	1.9	1.9
Acid-soluble	5.8	9.5	9.0	7.2	6.7	6.3	6.1	6.0
Holocellulose content	52.9	51.7	55.0	67.0	67.6	68.3	69.9	73.2
α-Cellulose content	27.5	27.0	29.3	31.8	34.1	32.4	38.3	38.6
Total sugar content	50.5	56.1	56.4	56.4	57.1	58.6	58.0	59.5
Starch content	5.6	6.2	5.5	4.4	4.1	2.3	2.5	1.9
Protein content	11.8	17.9	17.1	16.4	15.3	14.7	14.0	12.5
Phenolic acid content	0.23	0.46	0.40	0.32	0.37	0.40	0.56	0.64
Crystalline index	24.1	19.7	22.3	23.0	23.5	23.6	25.4	30.3

[a] Dried weight of material actually obtained (gr).

Table 2.1 (continued)

Component	Sliced sample number							
	No.14	No.16	No.18	No.20	No.22	No.24	No.26	No.28
Dried weight matter	2.4 (2.36)[a]	3.2 (2.20)	3.6 (2.47)	3.8 (2.58)	4.1 (2.80)	4.2 (2.87)	5.1 (3.49)	5.4 (3.68)
Lignin content								
Klason	2.1	1.3	2.0	2.7	3.3	3.3	1.9	3.2
Acid-soluble	5.3	5.0	4.9	4.8	4.5	4.2	3.5	3.9
Holocellulose content	73.4	71.1	75.8	78.0	83.5	82.3	82.0	83.7
α-Cellulose content	36.8	39.1	39.5	38.9	40.7	40.4	45.7	43.9
Total sugar content	60.2	66.9	67.7	74.7	73.5	74.5	75.0	79.5
Starch content	1.0	0.8	0.9	0.8	0.9	0.9	1.1	0.5
Protein content	13.4	11.6	11.5	11.7	11.9	11.5	11.3	10.1
Phenolic acid content	0.62	0.65	0.74	0.67	0.75	1.00	0.97	0.98
Crystaline index	30.0	30.9	35.1	34.4	36.5	35.5	40.4	41.5

[a] Dried weight of material actually obtained (gr).

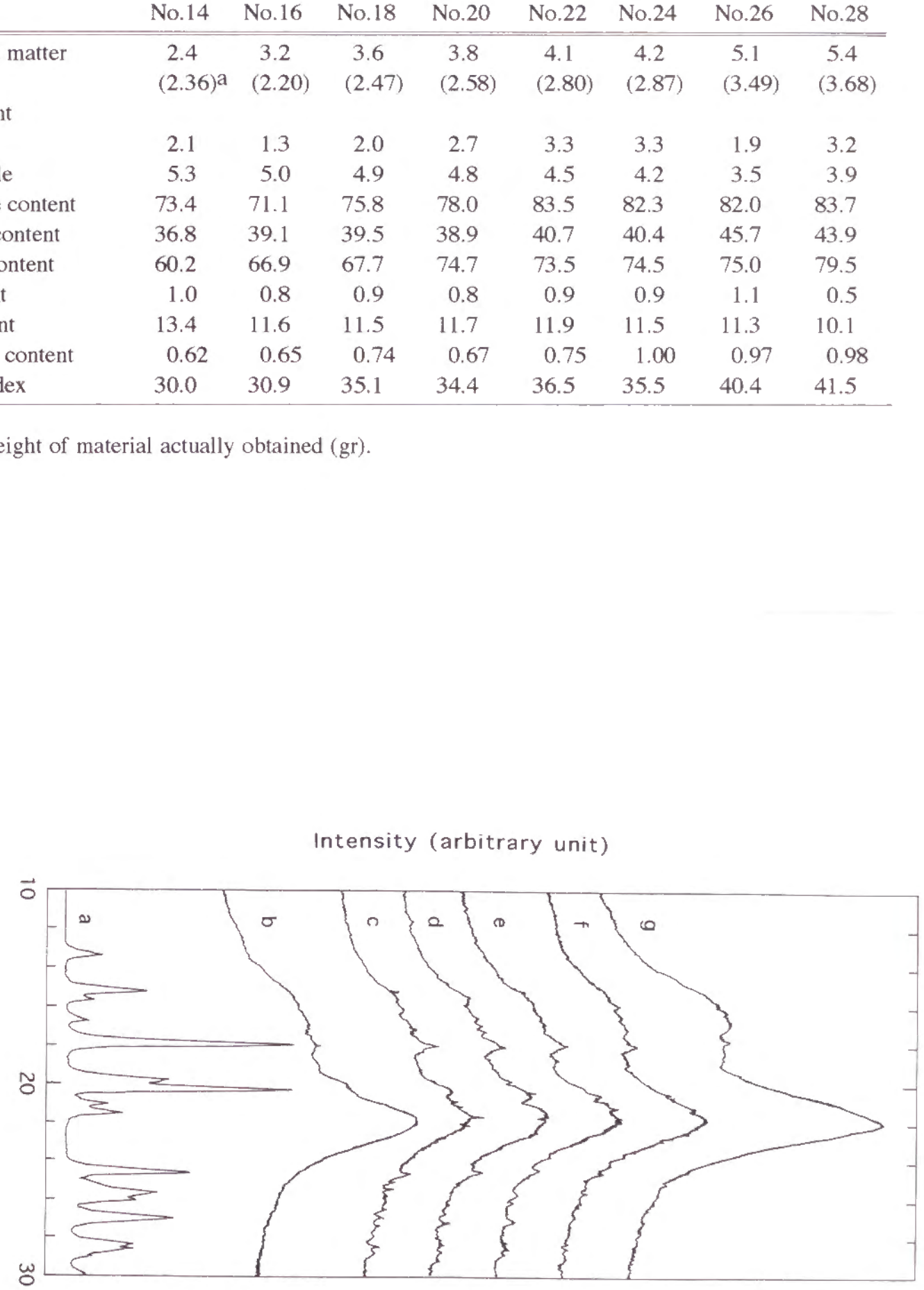

Fig. 2.3 X-ray diffraction patterns of six different portions of 17th internode and L-tyrosine.
(a), L-tyrosine;
(b), sliced sample No.1; (c), sliced sample No.2;
(d), sliced sample No.3; (e), sliced sample No.4;
(f), sliced sample No.14; (g), sliced sample No.28.

tendency. These results were also in good agreement with results of Itoh (1990).

It was concluded from the results that the internodal growth proceeded with height from the intercalary meristem located at the sliced sample No.2, in reverse direction from the overall growth of bamboo where downward progress of maturity was clearly demonstrated.

The differences in chemical composition of the sliced sample No.1 from that of the adjacent sample No.2 indicate that this portion included the intercalary zone to the node whose morphology was quite different from the internodal portion (Grosser and Liese, 1971), because this portion was in contact with the node separating the present 17th internode from the lower 16th internode.

2.3.2 Neutral sugar composition

As shown in Table 2.2, nearly 90 % of the neutral sugars were found to be glucose and xylose. At lower portions some glucose residues were originated from starch as shown in Table 2.1, while much of the glucose was from cellulose at upper portions. The major portions of xylose may be present as arabinoxylan (Higuchi, 1987). The ratio of arabinose to xylose decreased with maturation. The arabinose in the lower portions, however, may include residues attached to other polysaccharide or related components, *e.g.* arabinogalactan protein as described in Chapter 5, because of higher arabinose content than the arabinoxylan previously isolated from bamboo shoot (Maekawa and Kitao, 1973). The lowest xylose content with higher arabinose and galactose contents in the sliced sample No.2 indicated that the position of the intercalary meristem, where the internodal growth initiated, located in this sliced numbered position, 1.06 to 2.13 cm above the node.

Table 2.2 Neutral sugar composition of 17th internode of immature Mosochiku culm

(relative weight %)

Component	Sliced sample number							
	No.1	No.2	No.3	No.4	No.6	No.8	No.10	No.12
L-Rhamnose	0.8	0.7	0.6	0.6	0.6	0.6	0.7	0.3
L-Arabinose	5.4	6.0	4.7	3.6	5.1	4.2	4.0	3.9
D-Xylose	31.9	27.5	33.3	30.9	40.9	35.2	34.0	39.7
D-Mannose	2.0	1.4	1.2	1.1	0.7	0.7	1.1	0.6
D-Galactose	3.5	3.0	2.8	1.7	2.0	1.6	1.6	1.4
D-Glucose	56.4	62.4	59.3	62.0	50.6	57.8	58.5	54.1

Table 2.2 (continued)

Component	Sliced sample number							
	No.14	No.16	No.18	No.20	No.22	No.24	No.26	No.28
L-Rhamnose	0.7	1.1	1.1	0.8	1.0	0.6	0.5	0.2
L-Arabinose	5.5	6.7	5.5	4.0	5.3	4.3	3.5	2.5
D-Xylose	43.5	45.9	46.4	43.9	45.2	47.9	38.7	32.5
D-Mannose	0.7	0.6	0.7	0.5	0.1	0.6	0.5	0.3
D-Galactose	1.7	1.7	1.5	1.1	0.8	1.1	1.4	0.9
D-Glucose	47.7	43.9	44.9	49.7	47.5	45.5	55.4	63.6

2.3.3 Phenolic acid composition

p-Coumaric and ferulic acids, the major phenolic acids in bamboo cell walls, were divided into three types, free, soluble-bound and insoluble-bound, and the internodal variation of these phenolic acids were shown in Figs. 2.4 and 2.5. Both phenolic acids were exclusively extracted as a *trans* form. Among three types, insoluble-bound type was the main constituent and its contents was 10 times higher than the other types.

As for *p*-coumaric acid, all types increased with height in the portions higher than the sliced sample No.2. Free and soluble-bound types increased rapidly at upper one third portions (>No.20), whereas insoluble-bound type increased linearly in the portions between the slices from No.2 to 26. Irregularity at the sliced samples No.1 and No.28, both ends of the internode, may be due to an effect of the node which has been expected to have different composition because of its unique morphology (Grosser and Liese, 1971). Previously, *p*-coumaric acid was reported to esterify at γ-position of the side chain of lignin (Shimada *et al.*, 1971) and *O*-5 of arabinofuranosyl pendant of arabinoxylo-oligosaccharides (Ishii *et al.*, 1990c). A close correlation between the increase of the insoluble-bound *p*-coumaric acid and lignin contents rather than the arabinose content indicate that *p*-coumaric acid was mainly present as complexes with lignin. In addition, low values of methanol soluble *p*-coumaric derivatives (free and soluble-bound contents) preclude the presence of *p*-coumaric acid pool for lignin biosynthesis and suggest a quick incorporation into the lignin polymer.

Marked different profiles were observed in the ferulic acid contents. As shown in Fig. 2.5, insoluble-bound type increased with height up to the sliced sample No.12 (11.70-12.78 cm from the bottom) but had a plateau above this portion. Both the free and soluble-bound

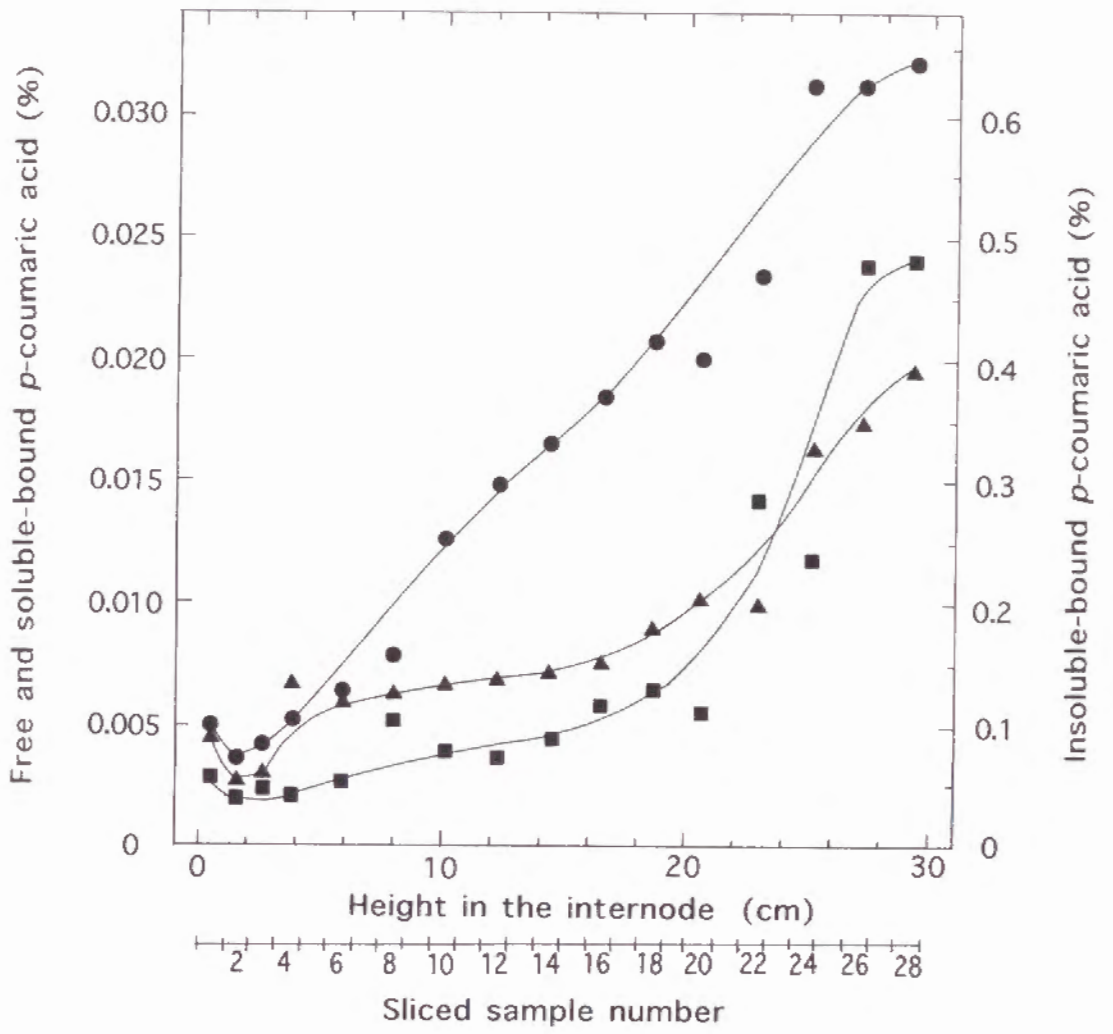

Fig. 2.4 Variation of three types of *trans-p*-coumaric acid contents.
▲, free type;
■, soluble-bound type;
●, insoluble-bound type.

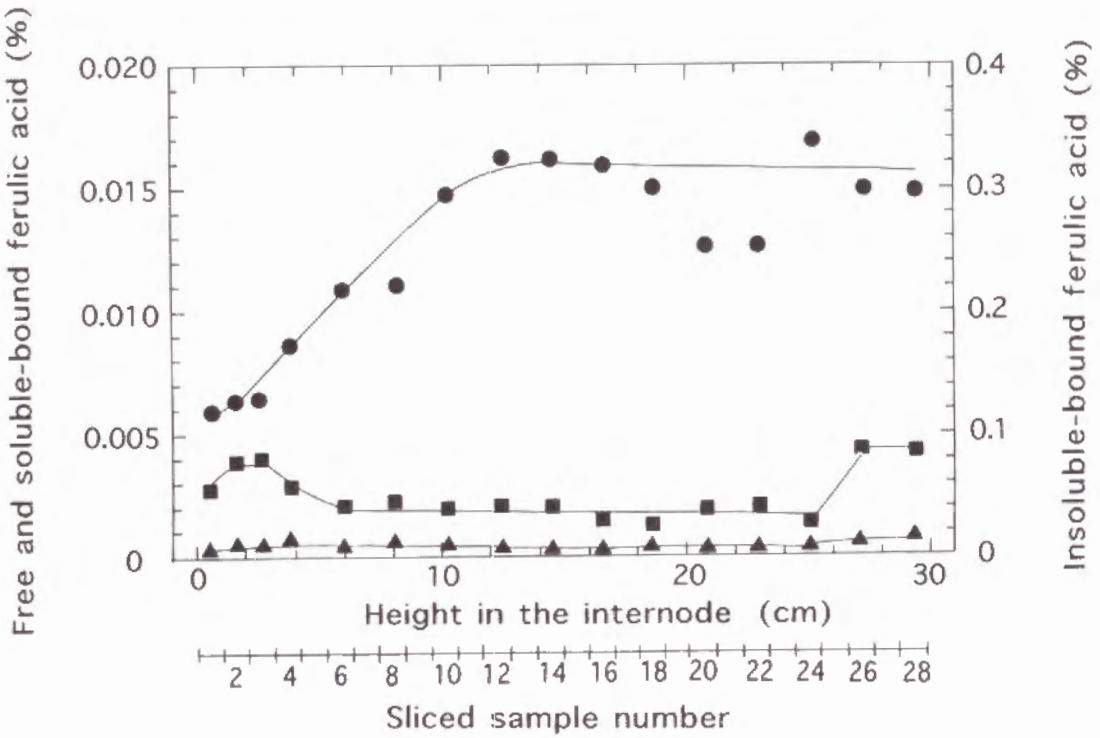

Fig. 2.5 Variation of three types of *trans*-ferulic acid contents
▲ , free type;
■ , soluble-bound type;
● , insoluble-bound type.

ferulic acid contents were very low and did not change throughout the internode except portions close to both ends. Ferulic acid has been considered to control cell elongation by forming diferuloyl bridges between arabinoxylan chains (Kamisaka *et al.*, 1990). If this is the case, bamboo cells will continue to elongate during the increase of the insoluble-bound ferulic acid content. In this sense, the elongation of cells is assumed to cease at a middle portion of the present internode.

2.3.4 Free amino acid composition

Sixteen free amino acids were detected on chromatograms given by amino acid analyzer, twelve of which are shown in Table 2.3. L-Proline and L-phenylalanine were not quantified because of their low content. Both L-leucine and L-isoleucine were also present but heavily overlapped with other amino acids, such as norleucine. The amino acid content has a tendency to decrease with ascending height above the sliced sample No.2. This tendency was the most prominent with L-tyrosine, which was the most abundant amino acid existed as a crystalline form in an immature culm of Mosochiku as described in the previous chapter. The straightforward correlation between decrease in the free L-tyrosine content and progress of maturity and lignification indicates the presence of tyrosine pool prior to initiation of lignification and localization of the intercalary meristem at the sliced sample No.2.

2.3.5 Crystallinity

Figure 2.3 shows X-ray diffractograms of the representative portions within one internode including the sliced sample No.1 to 4, 14 and 28, and crystalline L-tyrosine as a reference. At lower portions the diffractions due to L-tyrosine could be clearly detected and decreased gradually at upper portions. As expected from the L-tyrosine content, the sliced sample No.1 had a similar diffractogram as the sliced sample

Table 2.3 Free amino acids components of the 17th internode of immature Mosochiku culm

(mg / dried sample 100g)

Component	Sliced sample number							
	No.1	No.2	No.3	No.4	No.6	No.8	No.10	No.12
Asp	85.6	40.8	47.2	44.8	47.9	46.2	43.2	48.8
Thr	16.4	9.2	8.2	7.2	6.4	6.2	5.6	5.6
Ser	19.2	16.8	18.0	15.6	15.8	12.8	12.2	12.0
Glu	4.8	8.6	11.8	11.4	11.8	12.0	12.0	12.4
Gly	2.2	1.8	2.6	2.4	2.8	2.0	2.0	1.4
Ala	13.4	13.0	14.6	15.4	14.8	13.2	12.6	12.4
Val	7.8	6.6	7.2	7.2	7.8	7.0	6.6	6.8
Lys	4.0	4.2	4.4	4.0	3.8	3.8	3.0	3.2
His	3.4	3.2	3.2	2.8	2.8	2.8	2.4	2.4
Arg	4.2	4.0	4.2	3.8	3.8	3.2	2.0	2.0
γ-ABA	36.6	23.0	23.0	27.8	22.4	23.0	23.4	24.8
Tyr	247.8	900.4	898.6	860.2	774.0	699.9	638.2	627.2
Total	445.4	1031.6	1043.0	1002.6	914.6	812.4	763.2	759.0

Table 2.3 (continued)

Component	Sliced sample number							
	No.14	No.16	No.18	No.20	No.22	No.24	No.26	No.28
Asp	46.6	43.6	47.8	44.2	44.8	45.8	45.0	45.8
Thr	4.8	5.6	3.4	3.4	3.2	3.2	3.2	2. 6
Ser	12.4	12.0	12.4	11.4	13.0	11.8	11.4	11.6
Glu	12.0	11.4	13.8	12.0	11.4	10.8	10.2	11.6
Gly	1.6	2.2	1.6	1.4	2.2	1.4	1.4	1.4
Ala	12.6	12.6	13.8	12.8	13.8	13.8	13.8	13.8
Val	6.8	6.4	7.0	6.4	6.4	6.8	6.4	6.4
Lys	3.0	3.2	3.0	2.6	3.0	3.6	3.2	3.2
His	2.8	2.2	2.4	2.4	2.8	2.2	2.8	2.4
Arg	2.4	2.4	2.4	2.0	2.0	2.4	2.4	2.4
γ-ABA	27.2	22.6	27.8	26.2	27.4	28.0	24.4	26.2
Tyr	602.0	561.6	565.4	497.2	458.4	432.6	355.8	351.2
Total	734.2	685.8	700.8	622.0	588.4	562.4	480.0	478.6

No.28. Although the sliced sample No.2 and No.3 gave closely similar diffractograms, the former was slightly lower crystallinity than the latter, indicating a localization of the intercalary meristem in the sample No.2.

2.3.6 CP/MAS ^{13}C-NMR spectroscopic analysis

Figure 2.6 shows CP/MAS solid state ^{13}C-NMR spectra of the same representative portions within one internode as presented in the X-ray diffractograms (Fig. 2.3), together with that of crystalline L-tyrosine. The remarkable signals due to L-tyrosine, detected in the spectra of lower portions above the sliced sample No.2, gives the highest signal intensity. The intensity of the signals of L-tyrosine weakened with ascending height, while the signals appearing at 105.7, 75.8, 73.3 and 65.2 ppm were assignable to ring carbons of polysaccharides. The spectra of both ends of the internode (sliced sample No.1 and 28) agreed with lignocellulose with low lignin content (Kolodziejski *et al.*, 1982; Maciel *et al.*, 1985) and were similar to those of the lower half of 6 m-high immature culm as shown in the Fig. 1.3.

In view of this high sensitivity, solid state ^{13}C-NMR was applied to determine the position of the intercalary meristem. Although the ^{13}C spin-lattice relaxation times of L-tyrosine were different from those of polysaccharides, two close signals appearing at 105.7 ppm for polysaccharides and 119.0 ppm for L-tyrosine were selected, and the ratio of their intensities (intensity at 105.7 ppm / intensity at 119.0 ppm) was calculated. The contribution of lignin was not taken into consideration in this case because of its low content as shown in Table 2.1. Figure 2.7 shows a dependency between the ratios and the contents of L-tyrosine. Although the ratios were not identical to the values calculated from the contents of holocellulose (Table 2.1) and L-tyrosine (Table 2.3), the curve derived from the ratios gave a straightforward index for degree

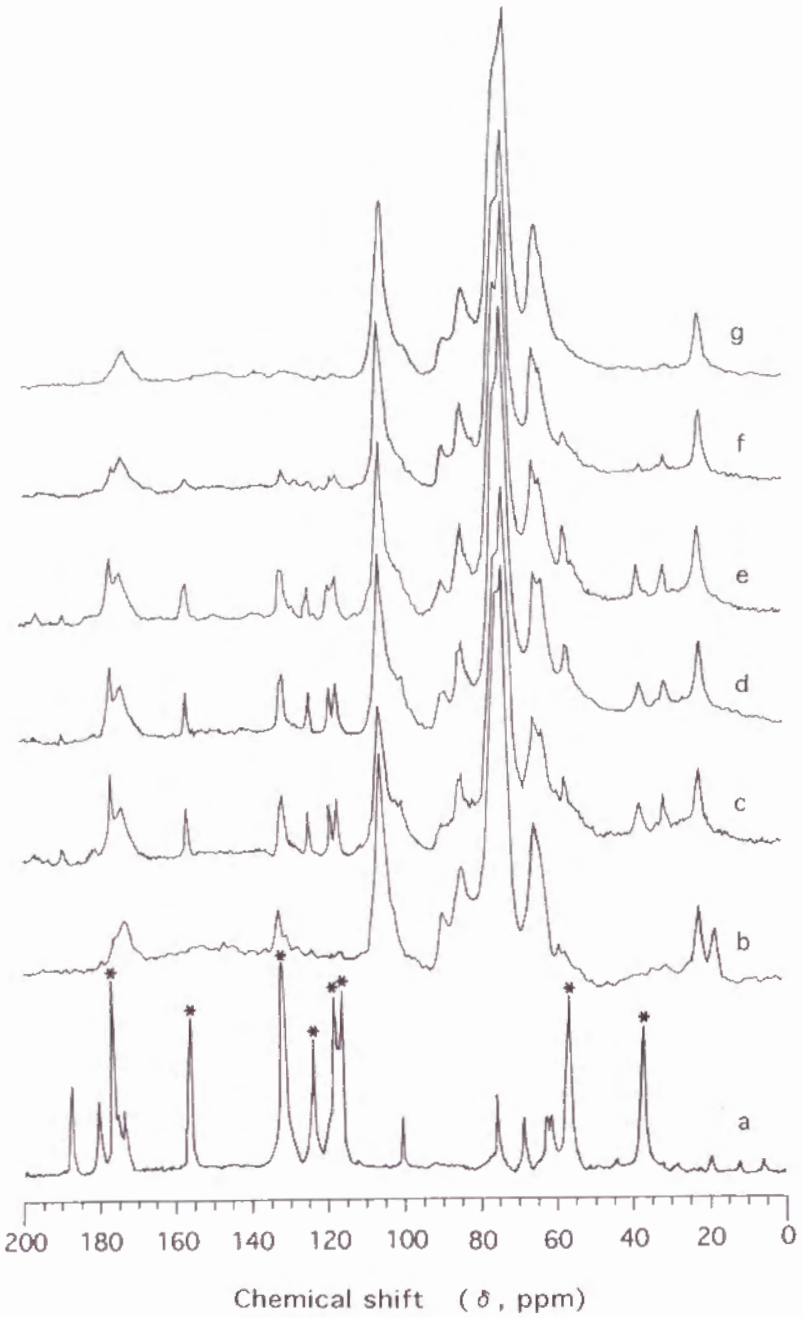

Fig. 2.6 CP/MAS ^{13}C-NMR spectra of six different portions of the 17th internode and L-tyrosine
(a), L-tyrosine;
(b), sliced sample No.1; (c), sliced sample No.2;
(d), sliced sample No.3; (e), sliced sample No.4;
(f), sliced sample No.14; (g), sliced sample No.28.
The signals shown by asterisks in (a) were due to L-tyrosine and all the other weak signals were of spinning side bands.

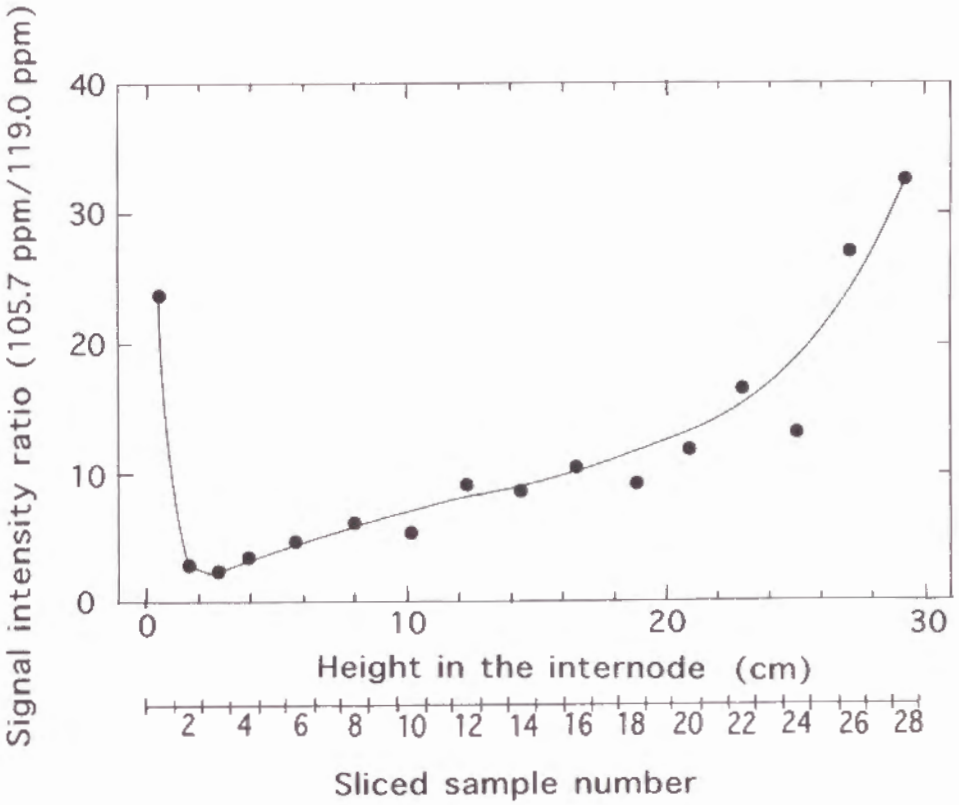

Fig.2.7 Relationship of ^{13}C-NMR signal intensities between sugar and L-tyrosine.

of maturity. Thus, the position of the intercalary meristem was found to be in the sliced sample No.2 which was localized at 1.06 to 2.13 cm above the lower end node.

2.4 Summary

Internodal growth of Mosochiku was characterized by analyzing chemical composition, X-ray diffraction and CP/MAS solid state ^{13}C-NMR. When the longest (17th) internode of an 6 m-high immature culm of Mosochiku, 29.8 cm in length, was sliced into 28 rings and subjected to the analyses, growth of Mosochiku was concluded to occur upward from the intercalary meristem localized at 1.06-2.13 cm above the node. CP/MAS solid state ^{13}C-NMR was proved to have a remarkable advantage to determining exact position of the intercalary meristem.

Chapter 3 Distribution of esterified phenolic acids in growing cell walls of immature Mosochiku culm

3.1 Introduction

In the previous two chapters, the vertical chemical composition changes of an immature Mosochiku culm having 6 m in height and of its longest internode were investigated. The most striking difference observed during elongation growth was in the content of bound phenolic acids. The *p*-coumaric acid content increased in good accordance with increase of the lignin content, whereas the ferulic acid content was inversely proportional to the lignin content. This indicates that the location and function of these two phenolic acids in Mosochiku are different. It has been suggested that *p*-coumaric acid was esterified at both γ and α positions of bamboo lignins in a ratio of 4:1 (Nakamura and Higuchi, 1976). Recently it was reported that several oligosaccharides esterified with *p*-coumaric and ferulic acids were isolated from enzymatic digest of bamboo shoot (Ishii *et al.*, 1990a, b, c, 1991). Polysaccharides esterified with ferulic acid have been a noticable component because of its controlling function of elongation growth (Fry, 1979, 1983; Kamisaka *et al.* 1990). In order to get more information about the function of phenolic acids in bamboo, a histochemical approach seems to be necessary.

Previous histochemical investigations of the phenolic acids in the graminaceous plants indicate that the esterified ferulic acid could be visualized specifically in the cell wall by its characteristic blue autofluorescence when it was irradiated with ultraviolet (UV) light and a bathochromic shift to green was noticed after exposure to ammonia or dilute alkali (Fulcher *et al.*, 1972; Harris and Hartley, 1976, 1980; Fincher, 1976; Hartley and Harris, 1981). UV microscopy has also been

effectively used for the distribution of lignin in tissues of wood (Musha and Goring, 1975) and bamboo (Itoh, 1990), and the phenolic acids in rice cell wall (He and Terashima, 1989). However, the distribution of these phenolic acids in bamboo tissue has not been clarified yet.

In this chapter, the distribution of esterified phenolic acids and lignin during whole and internodal growths of Mosochiku were investigated by using UV and fluorescence microspectroscopic methods.

3.2 Materials and methods

3.2.1 Materials

Two elongating culms of immature Mosochiku about 6 m in height were harvested for histochemical analyses.

One culm having 620 cm in height was harvested on May 16, 1989, and used to investigate the distribution of phenolic acid during whole growth. After bamboo-sheaths were pealed off, the culm was divided into 6 portions as described in 1.2.1. A small block was cut out from the lower part of the internodes which located at the middle height of each portion: No.1 from 7th, No.2 from 13th, No.3 from 18th, No.4 from 22nd, No.5 from 26th, No.6 from 32nd internode, respectively, as shown in Fig. 3.1.

Another culm having 589 cm in height was harvested on May 10, 1991, and used to investigate the internodal distribution of phenolic acids. After bamboo-sheaths were peeled off, 5 blocks were cut out from the longest internode, 17th internode with 29.6 cm in length (334.2-363.8 cm from ground), and designated as No.I to V from the bottom to the top as shown in Fig. 3.2.

3.2.2 Sample preparation for microscopy and chemical analyses

Each block prepared as described above was radially sectioned into two parts. One part was used for chemical analysis, and the other

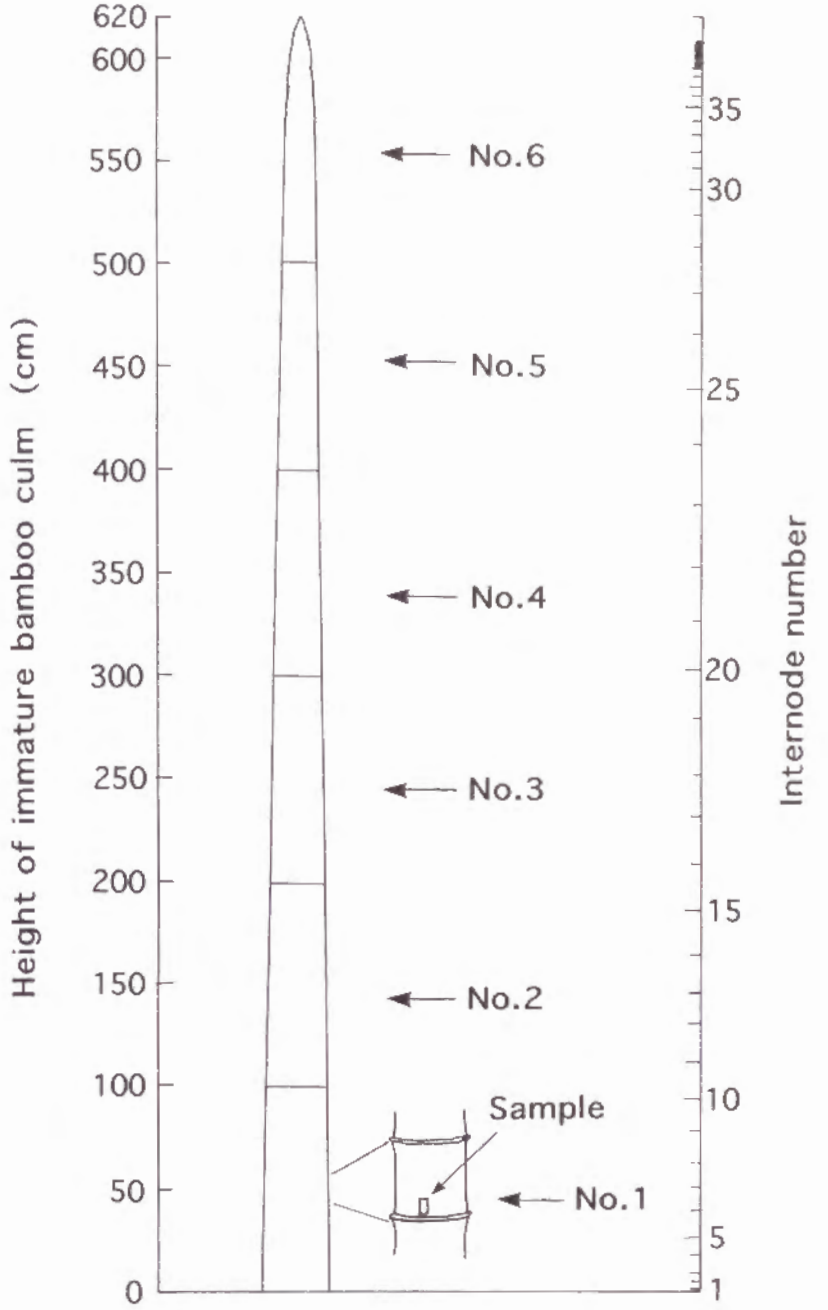

Fig. 3.1 Positions of six samples taken from an immature Mosochiku culm.

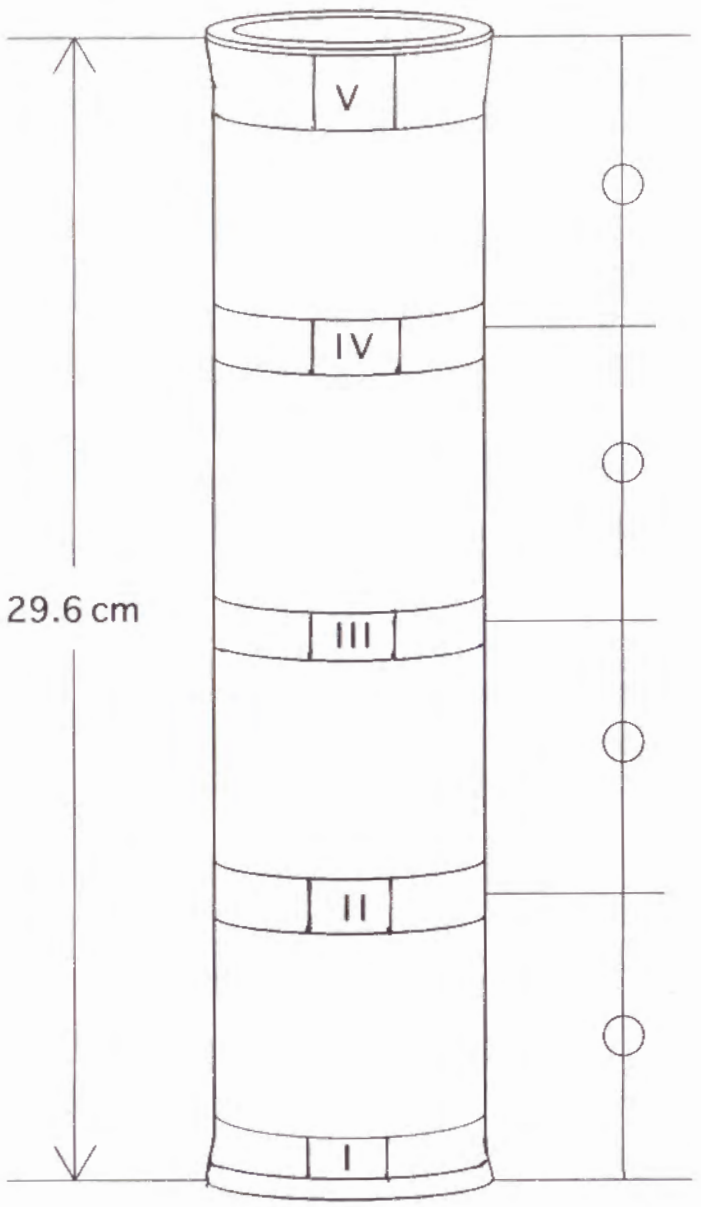

Fig. 3.2 Schematic illustration of positions of sample blocks taken from the longest internode (17th internode).

was tangentially sectioned into three portions. The middle portion of them was fixed with 3 % glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2). Transverse sections of 20 μm thickness were cut from the fixed blocks (No.1-3, No.I-V) on a sliding microtome with a frozen specimen stage for florescence microscopic analysis. Since the blocks of No.4-6 were fragile, 40 μm thick sections were cut. The rest of each block was dehydrated with a graded ethanol series and embedded in Luveak 812 resin. Thin (1 μm) sections for UV microscopic analysis were cut from embedded blocks with a Sorvall JB-4 microtome equipped with a glass or a sapphire knife.

Chemical analysis was carried out using a part of block after lyophilization and milling to pass 24-mesh screen by a Wiley mill. The contents of Klason lignin and esterified phenolic acids were determined as described in 1.2.2 and 1.2.5, respectively.

3.2.3 UV microspectroscopic spectrometry

Transverse thin sections were observed by a Zeiss UMSP80 microscope. Photographs were taken at 280 nm or 325 nm. UV absorption spectra were measured from 250 nm to 350 nm. Measurements were made at 5 nm band width and 1.5 μm spot diameter.

3.2.4 Fluorescence microscopic spectrometry

Transverse sections were observed with an epifluorescence microscope (BH-RFL, Olympus Kougaku Co.) with UV-excitation using four filters, UG1, DM400, L410 and L420. Microfluorescence spectra were measured by a Zeiss UMSP80 microscope. Illumination was carried out with three filters (BP 365, FT 395 and LP 397). Measurements were made at 20 nm band width and 5 μm spot diameter and the range was from 400 nm to 650 nm. Effects of alkali on

fluorescence spectrometry were determined by treatment with 0.1 M aq. ammonia on the sections.

3.2.5 UV and fluorescence emission spectroscopic analyses

For comparison, both UV and fluorescence emission spectra were measured with 7 compounds : L-tyrosine, *trans*-*p*-coumaric acid, ethyl coumarate, *trans*-ferulic acid, methyl ferulate, *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAX2) and milled bamboo lignin (MBL) containing 1.03 % *p*-coumaric acid and 0.13 % ferulic acid (w/w). The latter two were isolated from bagasse and Mosochiku by the methods of Kato *et al.* (1983) and Azuma *et al.* (1985), respectively. L-Tyrosine, *trans*-ferulic acid, *trans*-*p*-coumaric acid, were purchased form Nakarai Tesque Co., Ltd. Esters of phenolic acids were synthesized according to the method of Gubler *et al.* (1985). Measurements were made for neutral solution in 50 % aq. methanol or 1,4-dioxane, and their spectra were measured with a Shimadzu UV365 spectrometer and a Hitachi 850 spectro-fluorometer at 25 °C. Effects of alkaline on fluorescence spectra were determined by addition of ammonia to the above neutral solution to make 0.1 M aq. ammonia.

3.3. Results and Discussion

3.1 UV and fluorescence spectroscopic analyses of model compounds

Prior to UV and fluorescence microscopic analyses, UV and fluorescence spectra of 7 model compounds were measured. Results shown in Table 3.1 and the data reported previously (Nakamura and Higuchi, 1976; Harborne and Corner, 1961) indicate that esters of *p*-coumaric and ferulic acids could be differentiated each other by the position of λmax: the former shows an absorption maximum at 312 nm, while the latter at 325 nm. As expected from the phenolic acid

composition, MBL showed a typical UV spectrum of the *p*-coumaric acid ester (Nakamura and Higuchi, 1976; Azuma *et al.*, 1985). When the fluorescence spectra of the model compounds in neutral solution were compared with those obtained after alkaline treatment, the spectra of MBL and L-tyrosine showed only a slight bathochromic shift by 2-3 nm after alkaline treatment. The other compounds showed a larger bathochromic shift. Among them λmax of the feruloylated trisaccharide (FAX2) shifted to the longest wavelength, 475 nm.

Table 3.1 Characteristics of ultraviolet and fluorescence spectra of model compounds

Model compound	λmax (nm)		
	ultraviolet spectral data	fluorescence spectral data	
	neutral condition[a]	neutral condition[a]	alkaline condition[b]
L-Tyrosine	275	310	313
p-Coumaric acid	290	390	436
Ethyl coumarate	312	395	451
Ferulic acid	315	421	458
Methyl ferulate	320	426	455
FAX2 [c]	325	440	475
Milled bamboo lignin	312	456	458

a In 50% aqueous methanol except milled bamboo lignin which measurement was made in 50% aqueous 1,4-dioxane.
b In 0.1N NH_4OH.
c *O*-[5-*O*-(trans-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose

3.3.2 Microscopic analyses of six different portions of the immature culm

3.3.2.1 UV spectroscopic analysis

Figure 3.3 shows a series of UV micrographs taken at 280 nm of the 6 different portions. In these micrographs, three kinds of tissues, vessel, fiber and parenchyma were observed. Density of the UV absorptions became weaker from the bottom to the top with ascending height. At the bottom portion of the culm (No.1, 2), vessel and fiber cell walls showed dense UV absorption. At the middle portion of the culm (No.3, 4), fiber cells of outer bundle sheath showed substantial amount of UV absorption, and density of the UV absorption increased rapidly as approaching to the vessel cell wall. At No.5, only cell walls of vessel and fiber adjacent to vessel showed weak UV absorption. At the top portion of the culm (No.6), no distinctive UV absorption occurred in the cell walls, but it showed substantially a strong UV absorption probably due to proteins.

Following to microscopic analysis, UV absorption micro-spectrometric analysis was carried out to characterize chemical nature of UV positive compounds. Figure 3.4 shows variation of UV absorption spectra. As to the vessel cell wall, the absorption became weaker with ascending height. The absorption around 312 nm of No.1 seemed to be due to the *p*-coumaroyl group. With ascending height, the absorption at 325 nm due to feruloyl group appeared, while the absorption of lignin at 280 nm decreased (Fig. 3.4a). This indicates that vessel cell wall contained feruloyl groups at young stage of growth and accumulated *p*-coumaroyl group with progress of lignification. The results of chemical composition analysis are in good agreement with UV microscopic analysis shown in Table 3.2. In the case of the fiber cell

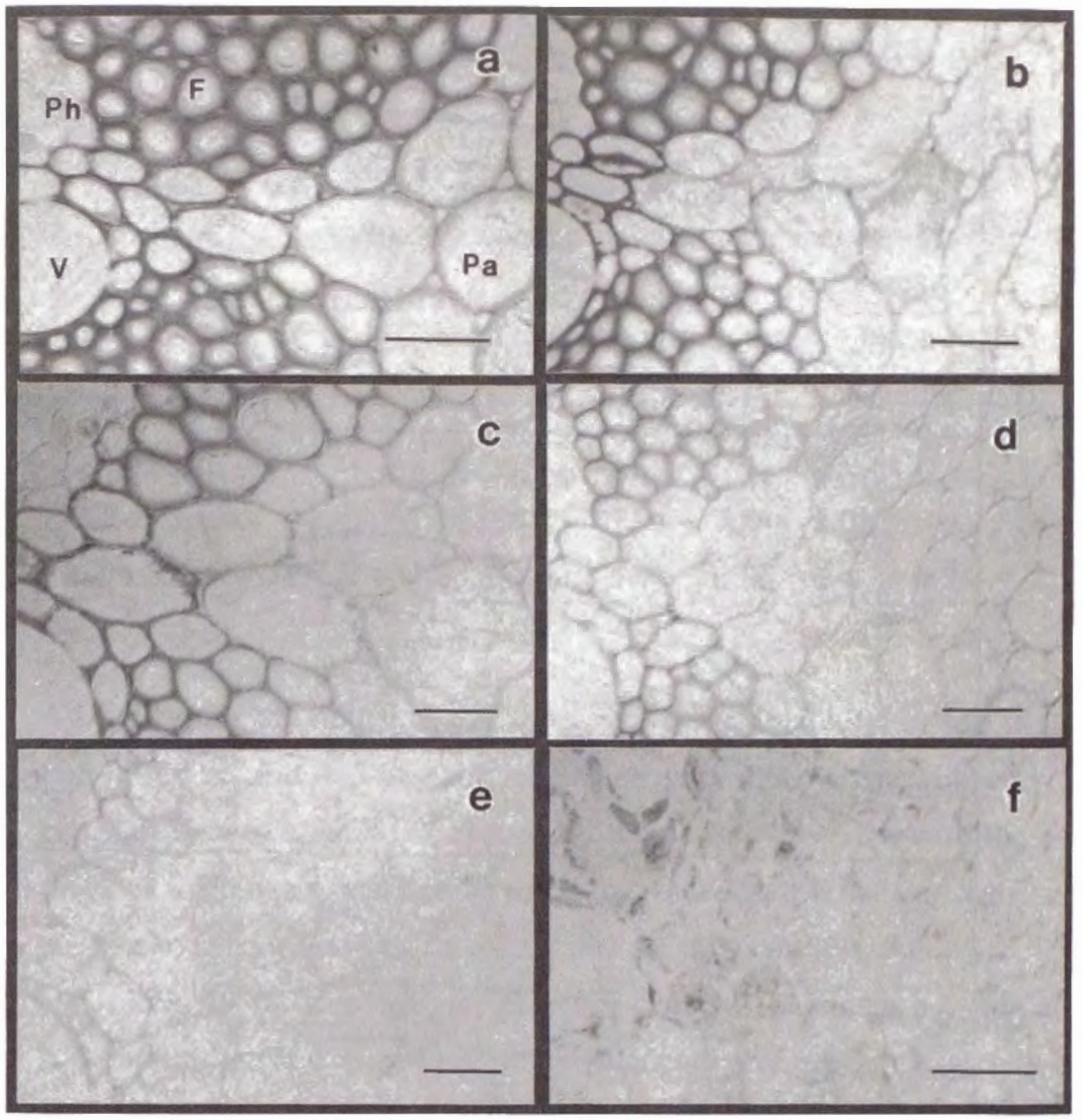

Fig. 3.3 Ultraviolet photomicrographs of six different portions of an immature culm.
Photomicrographs of (a) to (e) corresponded to the samples No.1 to 6, respectively.
V, Vessel; F, Fiber; Pa, Parenchyma; Ph, Phloem.
Scale bar: 20 μm.

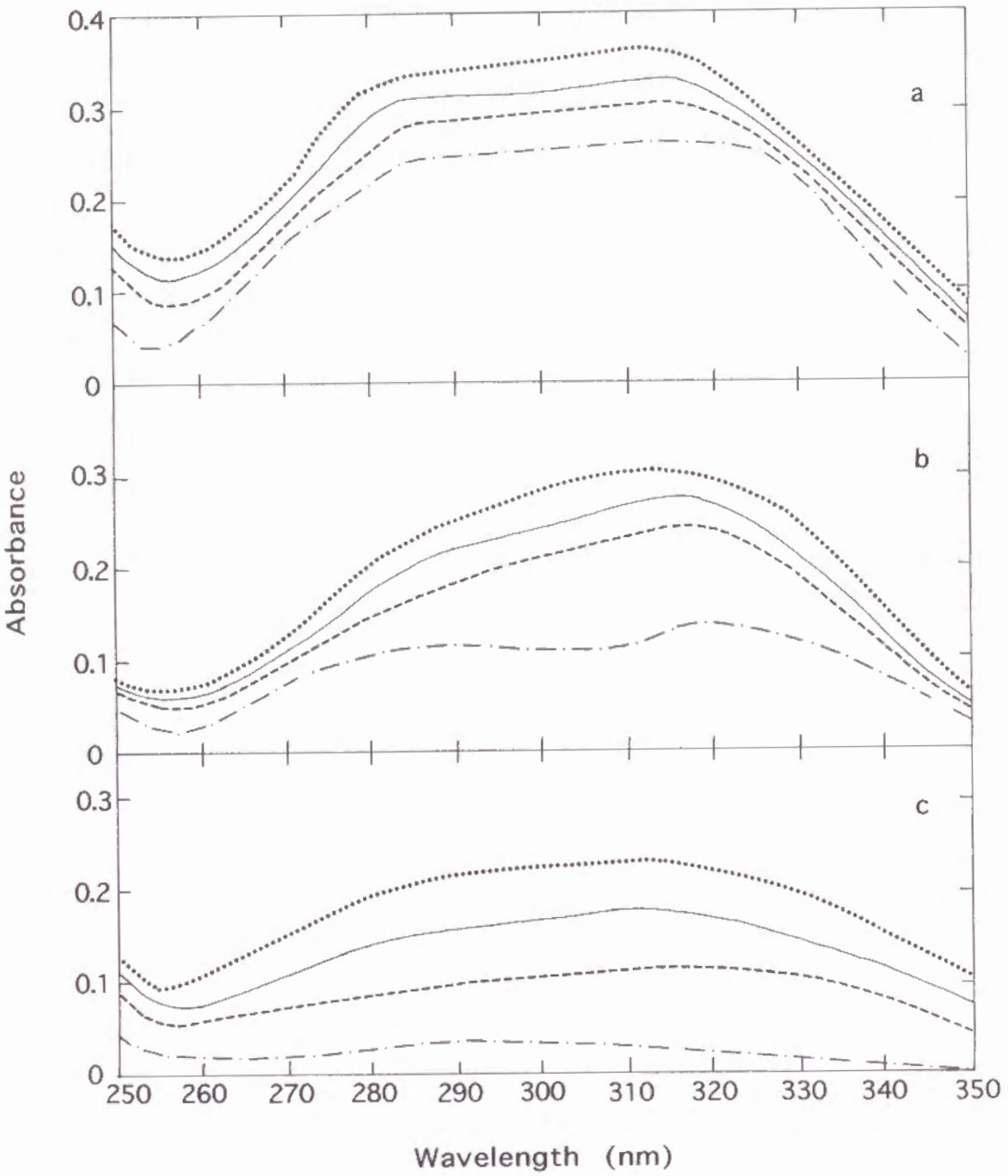

Fig. 3.4 Ultraviolet absorption spectra of vessel (a), fiber (b) and parenchyma (c) cell walls of different portions of an immature culm.
Spectra shown by (·····), (——), (- - - -) and (— · —) corresponded to the samples No.1, 2, 3 and 5, respectively.

wall (Fig. 3.4b), absorption near 320 nm was stronger than that at 280 nm and it was predominant at the lower portions of the culm (No.1-3).

Table 3.2 Variation of phenolic acid and lignin contents of six different portions in an immature culm

(weight %)

Compound	Positions of immature bamboo culm					
	No.1	No.2	No.3	No.4	No.5	No.6
p-Coumaric acid (C)	0.27	0.19	0.16	0.13	0.03	0.01
Ferulic acid (F)	0.18	0.14	0.14	0.24	0.07	0.06
Klason lignin	11.10	9.86	7.91	5.73	1.01	0.55
Ratio of (C) to (F)	1.5	1.4	1.1	0.5	0.4	0.2

A rather strong absorption near 320 nm shows that the fiber cell wall is rich in ferulic acid. In comparison to these cell walls, the parenchyma cell wall showed a weak UV absorption. This is in agreement with the previous finding that lignification of parenchyma cells did not start until the bamboo shoot grew up about 500-580 cm in height (Higuchi *et al.*, 1953; Itoh, 1990). However, the presence of broad maximum between 310-330 nm at No.1-3 indicates that esterification of parenchyma cell wall with phenolic acids proceeded slowly with growth. In contrast to the cell walls of vessel, fiber and parenchyma, degree of lignification and esterification with phenolic acids in the cell wall of phloem was concluded to be quite low because of extremely weak UV absorption. Previously, Yoshinaga *et al.* (1989) measured UV absorption spectra of various secondary cell walls in Mosochiku harvested in December of the same year after sprout. The present spectra of the vessel cell wall in the lower portion of the culm were similar to their result. However, absorptions at 280 nm and 310 nm in their spectra of fiber and

parenchyma, respectively, were much stronger than those in the present result. This indicates that deposition of lignin and phenolic acid in these cells proceeded in later stage of growth.

3.3.2.2 Fluorescence spectroscopic analysis

The fluorescence microscopy has been effectively used for examination of phenolic materials localization in graminaceous plant tissues (Fulcher *et al.*, 1972; Fincher, 1976; Harris and Hartley, 1976). Since the esterified ferulic acid with carbohydrate could be differentiated from the esterified *p*-coumaric acid by a bathochromic shift to longer wavelength observed with alkaline treatment as shown in Table 3.1, the distribution of the esterified phenolic acids in the bamboo tissue was further analyzed by fluorescence microscopy. When transverse sections from 6 different portions of the culm of Mosochiku were investigated with UV illumination, all cell walls of the immature bamboo tissue exhibited a blue autofluorescence due to lignin and esterified phenolic acids. At the upper portion of the culm (No.5, 6), the weak blue autofluorescence exhibited in all cell walls. With alkaline treatment, some alternation were observed : the blue color was changed into slightly greenish blue (Fig. 3.5a, b). This indicates that the ferulic acid esterified with carbohydrate was widely distributed in the young tissues of Mosochiku. This result is in agreement with that obtained by UV microspectroscopic analysis as shown in Fig. 3.3.

Figure 3.5 (c, d) shows the fluorescence photomicrographs of No.2 without and with the alkaline treatment. Different from No.5, a remarkable color change of vessel, fiber and parenchyma cell walls with the alkaline treatment were not observed. In contrast to these cells, only phloem cell wall was turned into bright green and this color was strengthened with descending height from No.5 to No.2. The intensity

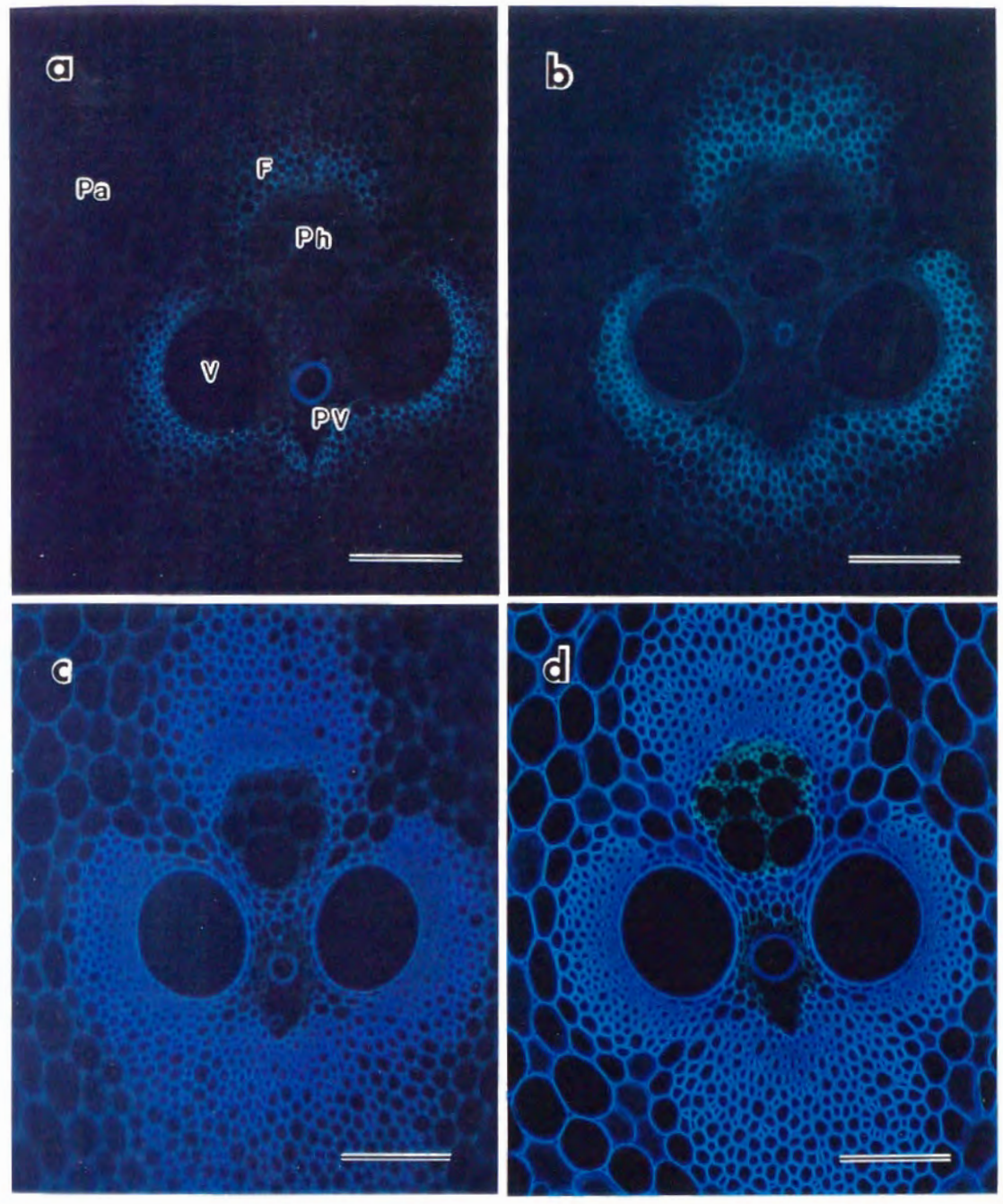

Fig. 3.5 Autofluorescence microphotographs of vascular bundles without (a, c) and with (b, d) alkaline treatment.
Two pairs of photographs [(a, b) and (c, d)] correspond to No.5 and No.2.
V, Vessel; F, Fiber; Pa, Parenchyma; Ph, Phloem;
PV, Protoxylem Vessel. Scale bar: 100μm.

of blue autofluorescence of No.2 was stronger than that of No.5. It may be due to lignin accumulation in cell walls.

In order to further analyze the bathochromic shift of No.2, microfluorospectra were measured at vessel, fiber and phloem cell walls. The results are shown in Fig. 3.6. The emission maxima of cell wall of fiber at the outer zone of vascular bundle, vessel and phloem shifted from 435 nm, 440 nm and 460 nm to 465 nm, 475 nm and 510 nm, respectively. The emission maximum shift of fiber cell wall was similar to that of model compound, FAX2 (Table 3.1). Based on the bathochromic shifts and the UV microspectra (Fig. 3.4), the esterified ferulic acid is concluded to be rich in fiber cell wall. Vessel cell wall also contained the esterified ferulic acid, but its fluorescence due to ferulic acid might be masked by contribution of lignin and *p*-coumaric acid. In the case of phloem cell wall, although color changed to green by the alkaline treatment, the emission maximum at 510 nm was different from that of FAX2.

3.3.3 Microscopic analyses of five different portions of the elongating internode

3.3.3.1 UV spectroscopic analysis

A series of UV micrographs taken at 325 nm of five different portions in elongating internode are shown in Fig. 3.7. A part of vascular bundle including vessel and its adjacent fiber were observed in these photographs. Density of the UV absorptions became stronger from the bottom (No.I) to the top (No.V). UV absorption was very weak at No.I and only the cell wall of protoxylem vessel showed a dense UV absorption. At the middle portions (No.II and III) the cell walls of vessel and fiber began to show UV absorption and the cell wall of parenchyma also showed a weak UV absorption. In No.IV and V, cells adjacent to phloem and the vessel showed substantial amount of UV

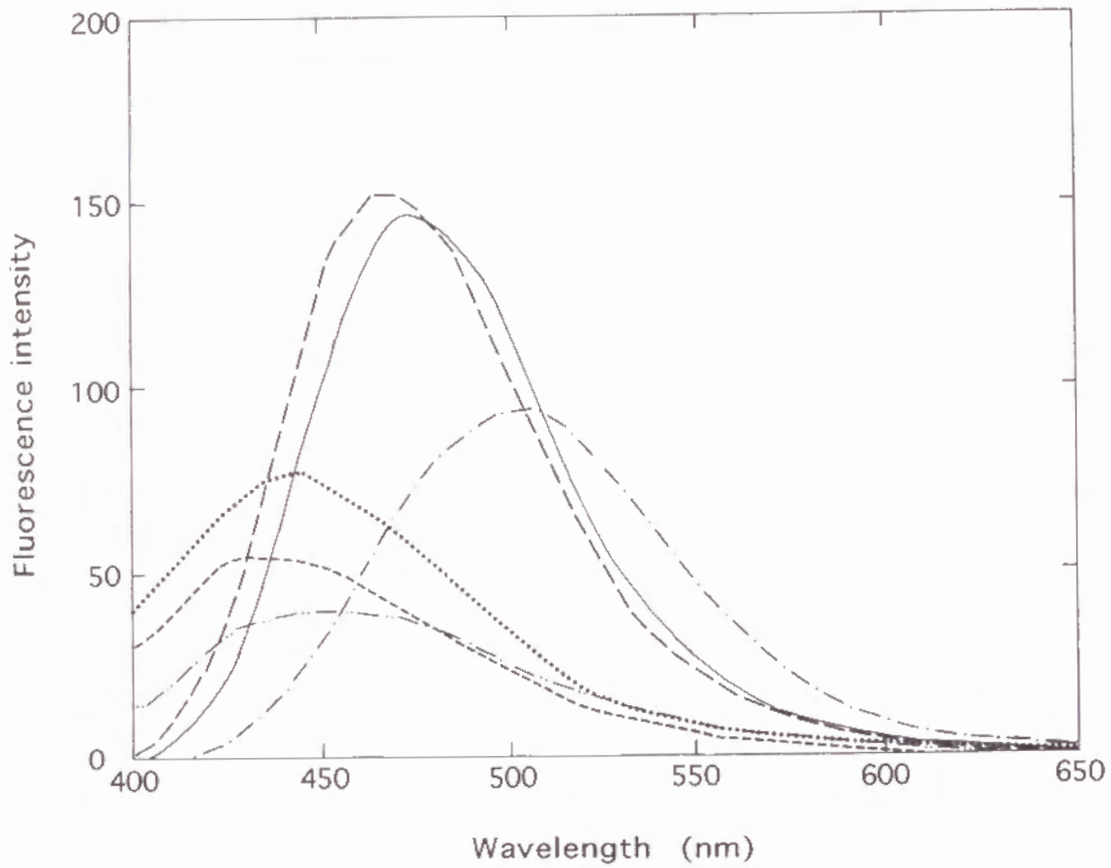

Fig. 3.6 Fluorescence microspectra of cell walls of vessel, fiber and phloem of the No.2.
Spectra shown by (-------) and (- - - -) correspond to vessel without and with alkaline treatment , (·····) and (——) represent corresponding spectra of fiber, and (—·—·—) and (— — —) represent corresponding spectra of phloem, respectively.

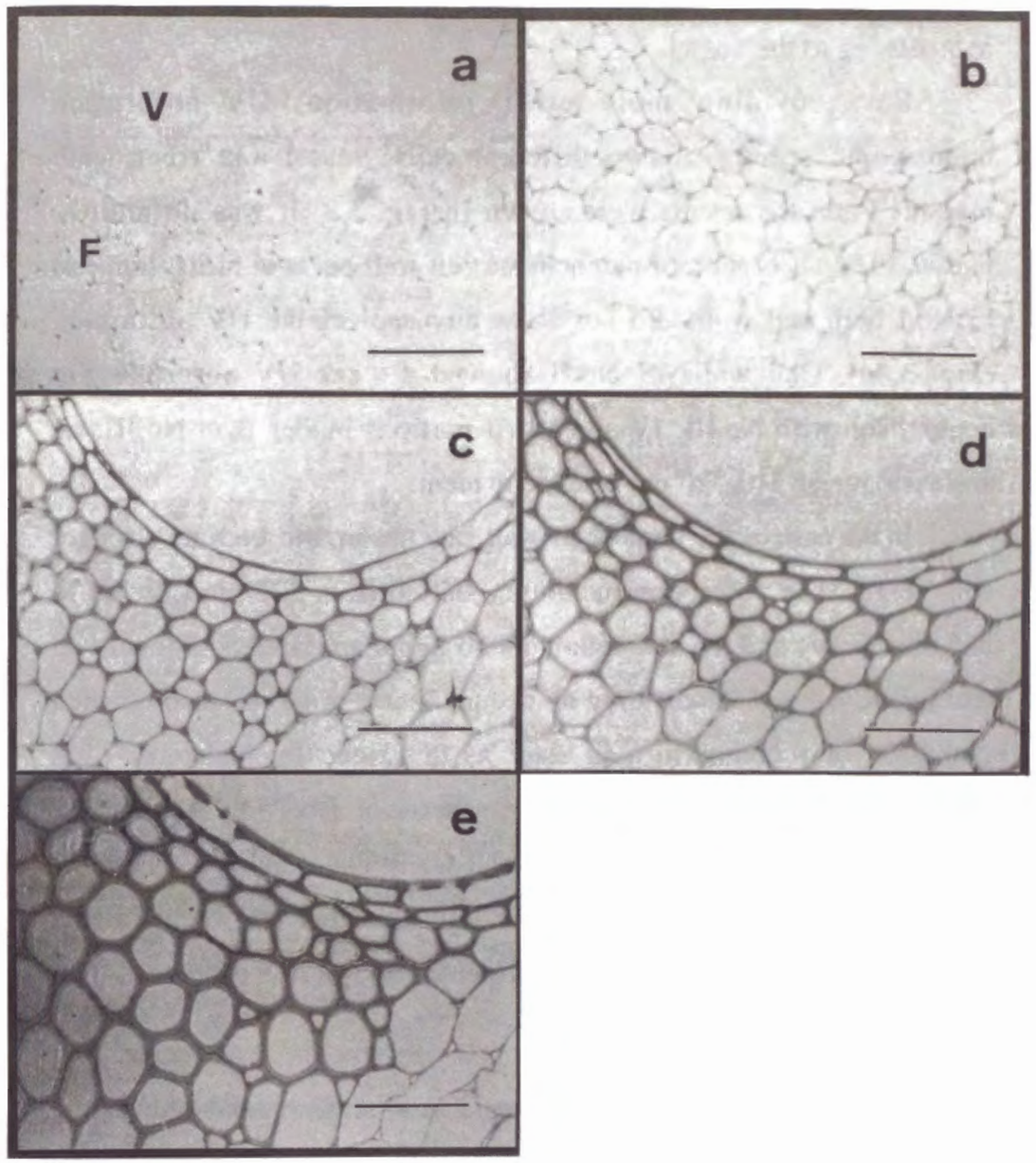

Fig. 3.7 Ultraviolet micrographs of vessel and fiber cell walls of five different portions of an elongating internode.
(a)-(e) correspond to No.I to V.
V, Vessel; F, Fiber. Scale bar: 20 μm.

absorption. The density of UV absorption increased rapidly as approaching to the vessel.

For providing more clear information, UV absorption microscopic spectra of two different cells, vessel and fiber were measured and the results were shown in Fig. 3.8. It was difficult to measure UV absorption of parenchyma cell wall because of its thinness. In No.I both cell walls did not show any appreciable UV absorption (Fig. 3.8a). Cell walls of No.II showed a weak UV absorption in comparison with No.III, IV and V. At portions higher than No.III, an absorption near 310-320 nm became evident.

In the case of the vessel cell wall, the absorption became stronger with height. The absorption maximum shifted from 325 nm (No.III) to 320 nm (No.V) and the absorption due to lignin appeared additionally at 280 nm with height, indicating an accumulation of lignin accompanied with that of *p*-coumaric acid ester as described by Nakamura and Higuchi (1976). The UV absorption spectra of fiber cell wall had a tendency similar to those of vessel cell wall. The results of chemical analysis in Table 3.3 indicate that the lignin content was higher at the upper portion. Based on the results presented above, it is concluded that

Table 3.3 Variation of phenolic acid and lignin contents of five different portions in an elongating internode

(weight %)

Compound	Portions of the 17th internode				
	No.I	No.II	No.III	No.IV	No.V
p-Coumaric acid (C)	0.04	0.17	0.35	0.41	0.68
Ferulic acid (F)	0.14	0.22	0.28	0.26	0.30
Klason lignin	1.2	1.4	2.1	2.7	3.2
Ratio of (C) to (F)	0.3	0.8	1.3	1.6	2.3

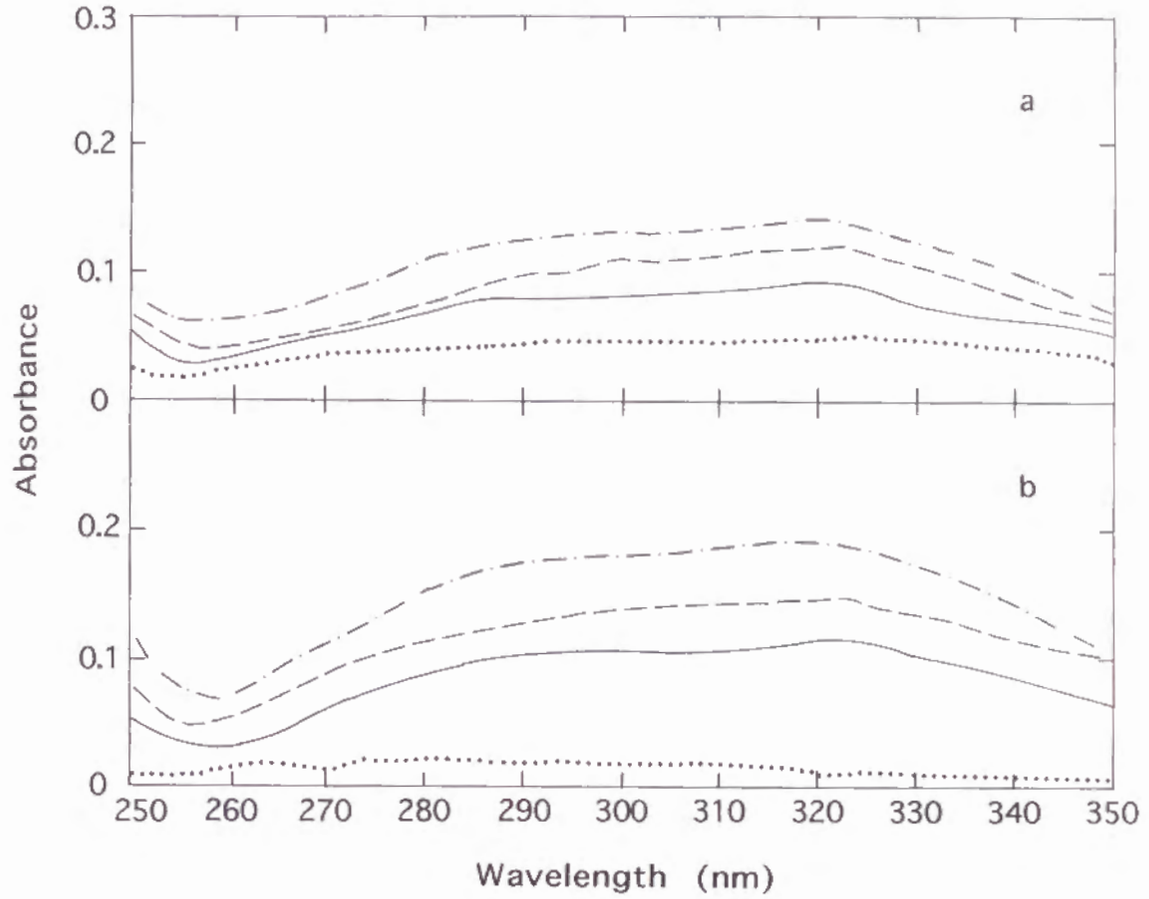

Fig. 3.8 Ultraviolet spectra of vessel (a) and fiber (b) cell walls of No.I to V of an elongating internode
Spectra shown by (·····), (——), (— —) and (— · —) corresponded to the samples No.II, III, IV and V, respectively.

the changes of spectra accompanying internodal growth and whole growth are similar, although the direction of maturation is different.

3.3.3.2 Fluorescence spectroscopic analysis

Figure 3.9a, c, e show fluorescence micrographs of vascular bundle of No.I, III, V without alkaline treatment, while Figure 3.9b, d, f show those with alkaline treatment. Fluorescence properties of No.II and No.IV were similar to those of No.III and V, respectively.

In the bottom portion (No.I), cell wall of protoxylem vessel showed a strong blue autofluorescence and the other cell walls exhibited weak one (Fig. 3.9a). After alkaline treatment, the color changed into slightly greenish blue (Fig. 3.9b), although this portion did not give clear UV absorption. To make clear this bathochromic shift of No.I, microfluorescence spectra were measured at protoxylem vessel, vessel, fiber and parenchyma cell walls (Fig. 3.10). The emission maxima of cell walls of protoxylem vessel, vessel and fiber shifted from 440 nm, 455 nm and 450 nm to 455 nm, 465 nm and 475 nm, respectively. In the case of parenchyma cell wall, the emission maximum was clearly detected at 460 nm at neutral condition, but quite a broad emission spectrum having a maximum at >460 nm was given after alkaline treatment. The shift of emission maximum of fiber cell wall was similar to the model compound, FAX2, indicating that the ferulic acids esterified with carbohydrate were widely distributed throughout the young internode tissues. As for No.III (Fig. 3.9c, e), cell wall of fiber at the outer zone of vascular bundle were blue and those at the inner layer were greenish blue without alkaline treatment. The chemical properties of the compounds which gave the greenish fluorescence was not clear at present. In No.V (Fig. 3.9e, f) the color change with the alkaline treatment was similar to that of the lower portion of immature bamboo culm (Fig. 3.5c, d).

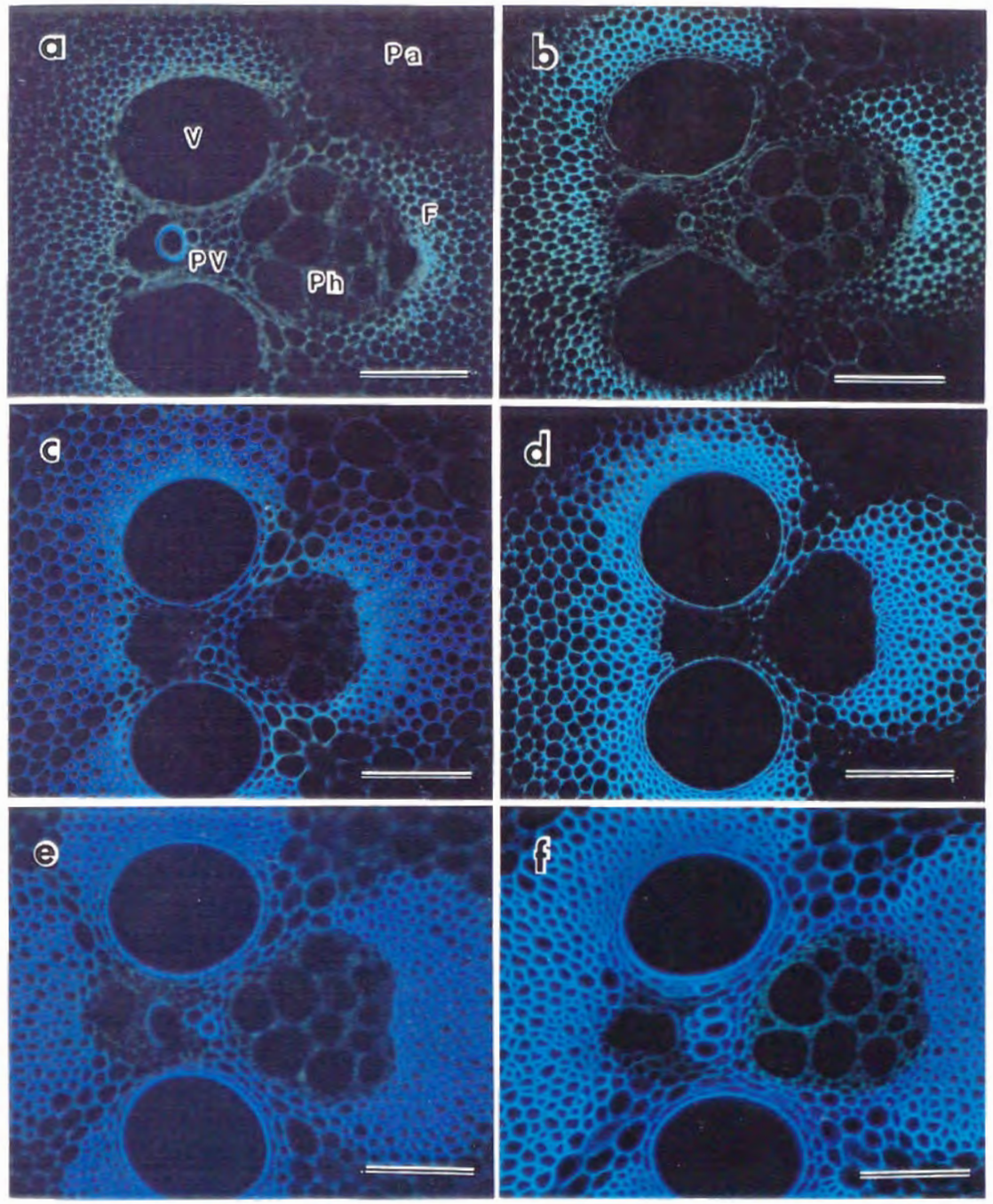

Fig. 3.9 Autofluorescence microphotographs of vascular bundles without (a, c, e) and with (b, d, f) alkaline treatment. Three pairs of photographs [(a, b), (c, d) and (e, f)] correspond to No.I, III and V.
V, Vessel; F, Fiber; Pa, Parenchyma; Ph, Phloem; PV, Protoxylem Vessel. Scale bar: 100μm.

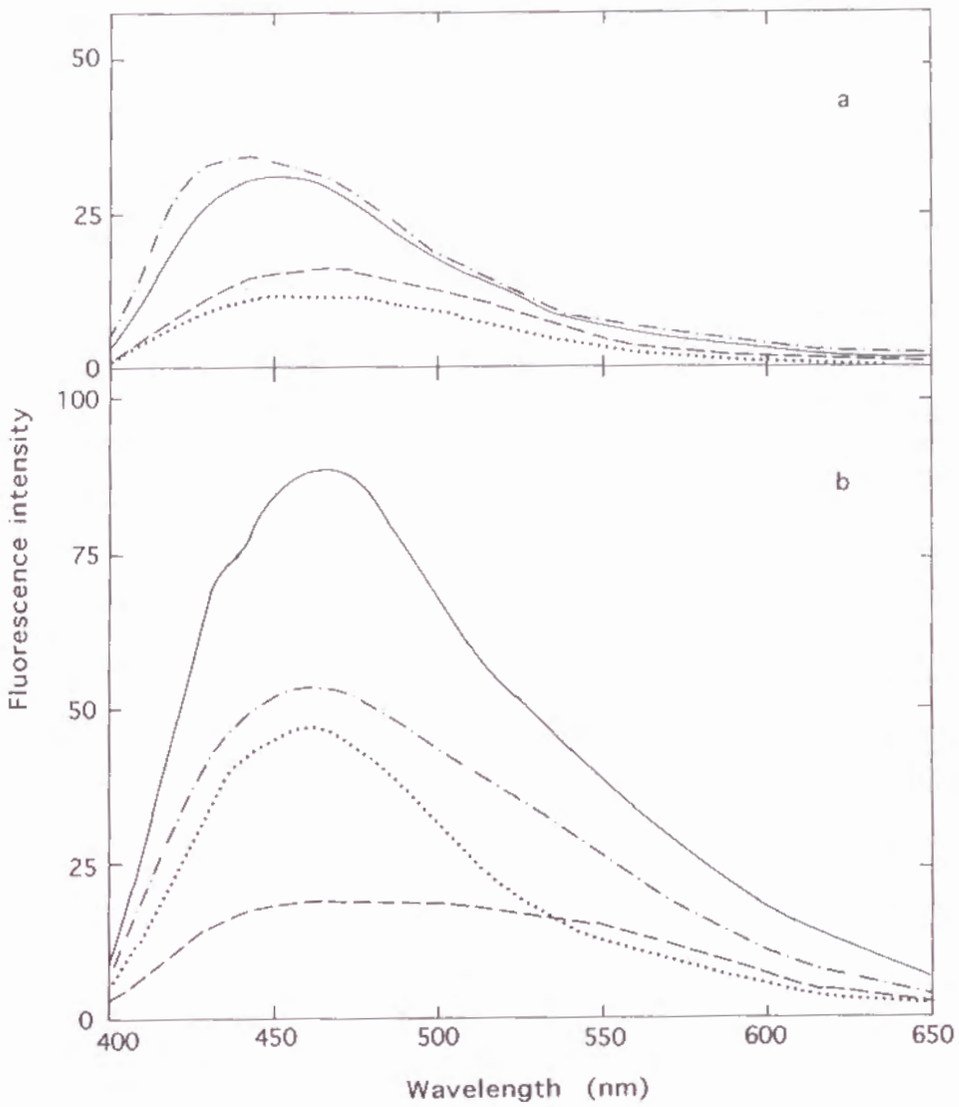

Fig. 3.10 Fluorescence microspectra of cell walls from No.I with (b) and without (a) alkaline treatment.
Spectra shown by (·····), (——), (– – –) and (— · —) corresponded to vessel, fiber, parenchyma and protoxylm vessel.

3.4 Summary

Variations of the distribution of esterified ferulic and *p*-coumaric acids during whole and internodal growth of Mosochiku investigated by UV and fluorescence microscopic techniques. The results indicate that the esterified phenolic acids were widely distributed throughout the tissue of immature Mosochiku having 6 m in height, but some distinctive differences in distribution of both acids were detected. An esterified ferulic acid was synthesized at the early stage of growth and accumulated earlier than *p*-coumaric acid and lignin. An esterified *p*-coumaric acid, in contrast, increased with progress of lignin accumulation. The accumulation of esterified phenolic acids and lignin progressed from inner to outer zone of vascular bundle. Although the direction of maturation is different, the change of distribution of esterified ferulic and *p*-coumaric acids accompanying internodal growth are concluded to be similar to the case of whole growth.

Chapter 4 Changes in properties of dimethylsulfoxide-soluble polysaccharides containing phenolic acids during whole growth of immature Mosochiku culm

4.1 Introduction

In the preceding chapters, the detailed chemical composition changes of an immature Mosochiku culm were characterized, and an immature culm of bamboo having 6 m in height was found to be appropriate to investigate its growth. One of the notable obtained features was in the differences of distribution profiles of ferulic and *p*-coumaric acids : *p*-coumaric acid increased from the top to the bottom of the culm in relation to the lignin content, while a reverse relationship was observed in the ferulic acid. Since these phenolic acids exist mainly as an insoluble-bound type and bamboo cells continue to elongate during increase of the insoluble bound ferulic acid content, further study on variation of macromolecules containing ferulic acid during growth of bamboo seems to be necessary to investigate growth mechanism of Mosochiku. Because dimethylsulfoxide (DMSO) is a mild solvent and usable to extract acetylated hemicelluloses without deesterification (Hägglund *et al.*, 1956), the author considered that polysaccharides esterified with phenolic acids could be extracted with DMSO. Maekawa and Kitao (1973) isolated a mixture of polysaccharides from a bamboo shoot by extraction with DMSO, but they did not mention about the existence of phenolic acids in the extracts and possible changes in their chemical properties during growth.

Incidentally from enzymatic digest of bamboo shoot of Mosochiku, two feruloylated oligosaccharides were isolated and their structures were characterized (Ishii and Hiroi, 1990a, b). Since the feruloyl residues undergo an oxidative coupling to form diferuloyl cross-linkages between polysaccharides (Geissman and Neukom, 1973), such coupling has been

considered to play an important role in cell wall extensibility and cell growth (Fry, 1979, 1983; Kamisaka *et al.*, 1990). The origin of these oligosaccharides is, however, remained to be solved.

In this chapter, by using DMSO as an extraction solvent native polysaccharides esterified with phenolic acids were isolated from six portions different in height of an immature culm of Mosochiku having 6 m in height, and their chemical properties were characterized in relation to its maturity. In addition, a feruloylated native arabinoxylan was isolated from the lowest 1 m-portion of the culm and characterized the position of feruloylation by identification of a known feruloylated arabinoxylobiose (FAX2) from its enzymatic digest.

4.2 Materials and methods

4.2.1 Extraction of DMSO-soluble polysaccharides containing phenolic acids

An immature culm of Mosochiku 6 m in height was harvested and cut into six 1 m-portions. Each portion was designated as No.1 to No.6 from the bottom to the top, and milled to pass 24 mesh screen as described in 1.2.1. Each bamboo powder was extracted with a mixture of ethanol-benzene (1:2, v/v), and each extractive-free powder (100 g) was extracted with 2 *l* of cold water for 24 hr and depectinated with 2 *l* of 0.5 % aq. ammonium oxalate at 40 °C for 24 hr. The residue was extracted twice with 2 *l* of DMSO at room temperature for 24 hr. The extracts were gathered, dialyzed against water, evaporated to a small volume and poured into 3 vol. of ethanol. The precipitate was recovered by centrifugation and dried *in vacuo* after solvent exchange to give DMSO-soluble polysaccharides containing phenolic acids.

4.2.2 Determination of esterified phenolic acids

Esterified phenolic acids were extracted with ethyl ether after saponification with 2 N sodium hydroxide and acidification with 6 N hydrochloric acid as described in 1.2.4. Acetovanillone was used as an internal standard. After solvent was removed by evaporation, phenolic acid composition was analyzed by HPLC (Waters M600, Div. of Millipore) with a column of YMC-Pack ODS-A-312 (10 x 150 mm). Elution was carried out at a flow rate of 1.0 ml / min, with a linear gradient from 100 % of A [water : methanol : acetic acid = 70 : 30 : 1 (v/v/v)] to 100 % of B [water : methanol : acetic acid = 50 : 50 : 1 (v/v/v)] in 30 min at room temperature. Elution was monitored by measuring absorbance at 325 nm (Lamda-Max 481, Waters, Div. of Millipore).

4.2.3 Neutral sugar and methylation analyses

Neutral sugar composition was analyzed as alditol acetates after hydrolysis with 1 N sulfuric acid for 6 hr described in 1.2.4. Methylation of the sample was carried out according to the method of Hakomori (1964) with slight modification (Sanford and Connrad, 1966) followed by the method of Kuhn and Terischmann (1963) The methylated sample was hydrolyzed with 90 % formic acid - 0.5 N sulfuric acid as described by Lindberg (1972). The hydrolysate was converted into partially methylated alditol acetates which were separated by GLC (Shimadzu GC-15) and analyzed by GC-MS (Shimadzu QP-2000) on a column of CBP-1 column (0.25 mm x 25 m) with the following temperature program: Column temperature for GLC was initially kept at 150 °C, linearly increased at 0.5 °C / min to 180 °C, then increased at 5 °C / min to 210 °C; Column temperature for GC-MAS was initially kept at 120 ℃, increased at 2 °C / min to 180 °C, kept at this temperature for 10 min, and increased again at 5 °C / min to 210 °C.

4.2.4 Determination of molecular weight

Weight average molecular weights ($\overline{Mw}$) were estimated by size exclusion chromatography using a column of Toyo-Pearl HW-75 (1.5 x 100 cm). The sample was solubilized in 80 % aq. DMSO and applied to the column which was eluted with the same solvent at room temperature. Elution was carried out at a flow rate of 0.875 ml / min. Elution of the polysaccharide was monitored by taking out 0.5 ml from each fraction and development of the color by the phenol-sulfuric acid method (Dubois *et al.*, 1956). Distribution of esterified phenolic acids was also monitored by measuring absorbance at 325 nm. The pullulans having known molecular weights (Shodex standard P-82) were obtained from Showa Denko Co., Ltd. (Tokyo, Japan) and used as standards.

4.2.5 Glucoamylase treatment of DMSO-soluble polysaccharides containing phenolic acids

Each DMSO-soluble polysaccharide containing phenolic acids (15-20 mg) was incubated with 75 units of glucoamylase from *Rhizopus niveus*, which was from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), at 40 °C for 3 hr and boiled in a water bath for 10 min to stop the reaction. The hydrolysate was dialyzed against distilled water and the material inside the dialysis tubing was lyophilized.

4.2.6 Identification of a feruloylated arabinoxylobiose

The DMSO-soluble polysaccharide containing phenolic acids from No.1 (300 mg) was incubated with Driselase (Kyowa Hakko, Co., Ltd.) partially purified by gel filtration on Sephadex G-50 at 36 °C for 24 hr and boiled in a water bath for 10 min to stop the reaction. The hydrolysate was poured into 3 vol. of ethanol. The supernatant was recovered by centrifugation and concentrated to dryness. The sample was solubilized in a small amount of water and applied on a column of Sephadex LH-20 (9.3 x 175 mm) equilibrated with water. The column was eluted with water to

remove unadsorbed material. Adsorbed material was then eluted with 50 % aq. 1,4-dioxane. The eluates were monitored by measuring absorbance at 325 nm for esterified phenolic acid and 480 nm for carbohydrate after development of color by the phenol-sulfuric acid method as described above. A fraction of adsorbed material was analyzed by HPLC on an analytical column of YMC-Pack ODS-AQ-303 (4.6 x 250 mm) using a mixed solution of water : methanol (1:1, v/v) as an eluent and a flow rate of 0.4 ml / min. A major feruloylated oligosaccharide of this fraction having retention time of 12.29 min at its center by HPLC on an analytical column was isolated by using a semipreparative column (YMC-Pack ODS-SH-343-5AQ, 20 x 250 mm) of the same type. The purity of the isolated feruloylated oligosaccharide was checked by analytical HPLC described above and TLC on Silica gel 60 (Merk No.5744) using 1-butanol : acetic acid : water (16 : 15 : 23, v/v/v) as a solvent to have Rf 0.54. The molecular weight of the isolated feruloylated oligosaccharide was determined in the negative-ion mode by LC/MS (Hitachi M-2000) with air pressure ionization (API) equipment using a column (6.0 x 150 mm) of YMC A-312 and a mixed solution of water : methanol (3:2, v/v) as an eluent. Flow rate was kept constant at 1 ml/min. An authentic feruloylated arabinoxylobiose, *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAX2), used as a standard as described in 3.2.5.

4.2.7 Spectroscopic analyses

IR spectra were measured with a Shimadzu FTIR-4000 spectrometer as potassium bromide discs. UV spectra were measured in a mixed solvent of water : DMSO (1:1, v/v) with a Shimadzu UV-365 spectrophotometer. The optical rotation of sample dissolved in the same solvent was measured with a JASCO DIP-4 differential polarimeter at 20 °C. ^{1}H- and ^{13}C-NMR spectra were recorded with a Brucker ARX-500 spectrometer (500.113 MHz for ^{1}H and 125,77 MHz for ^{13}C) in a mixed solvent of D_2O : DMSO-

d_6 (3:17, v/v) for native arabinoxylans containing phenolic acids at 70 °C and with a Brucker AM-300 spectrometer (300.1 MHz ^{1}H and 75.5 for ^{13}C) in D_2O for the isolated feruoylated oligosaccharide. Chemical shifts were referenced with TSP-d_4. H,H-COPY. H,C-COSY and H,C-COLOC experiments were carried out using standard Brucker soft ware.

4.3 Results and Discussion

4.3.1 Properties of the DMSO-soluble polysaccharides containing phenolic acids

Yield and chemical properties of the DMSO-soluble polysaccharides containing phenolic acids from 6 different portions of the immature Mosochiku culm are summarized in Table 4.1. The results showed that the extracted materials were mainly composed of native acetylated polysaccharides containing a small amount of phenolic acids. The major neutral sugar was xylose in the lower portions (No.1 and No.2), while the content of glucose increased with height. Proportions of the other neutral sugars, mannose, galactose and arabinose, were so low as the total of them amounted less than 10 %. Since native xylan in bamboo has been known as partial acetylated arabinoxylan, and taking the results of Maekawa and Kitao (1973) into consideration, present DMSO-extracts may be composed of a mixture of partially acetylated arabinoxylan, arabinogalactan and glucan. Based on the highly positive specific optical rotation at the top portion (No.6) as shown in Table 4.1 together with digestibility of more than 77-99 % of glucan by glucoamylase (data not shown), glucan was concluded to be mainly α-glucan such as starch.

Phenolic acids were composed of bound ferulic and *p*-coumaric acids. When both phenolic acid contents were plotted against the arabinoxylan content, only ferulic acid showed straightforward correlation with the arabinoxylan content as shown in Fig. 4.1. These results support

Table 4.1 Yields and properties of DMSO-soluble polysaccharides containing phenolic acids

	Positions of immature bamboo culm					
	No.1	No.2	No.3	No.4	No.5	No.6
Yield (%)	0.9	1.2	1.5	3.7	4.8	5.2
$[\alpha]_D^{25}$ (Degree)	-38.7	-27.0	-15.3	-21.8	-3.3	+75.5
Acetyl group content (%)	7.1	6.5	5.3	4.7	3.4	1.5
Uronic acid content (%)	3.0	3.2	2.7	2.8	2.2	1.7
Neutral sugar composition (%)						
D-Mannose	0.3	0.3	1.7	0.7	0.5	0.9
D-Galactose	1.7	1.8	3.0	3.3	2.8	3.3
L-Arabinose	4.4	3.9	3.8	3.1	3.1	2.7
D-Xylose	82.4	74.8	59.0	63.6	30.7	14.4
D-Glucose	11.1	19.2	32.5	29.3	63.0	78.7
Phenolic acid content (%)						
p-Coumaric acid	0.49	0.31	0.25	0.22	0.07	0.02
Ferulic acid	1.36	1.22	0.83	0.75	0.44	0.12
Arabinose / Ferulic acid (molar ratio)	3.71	3.71	5.46	7.58	8.53	28.1

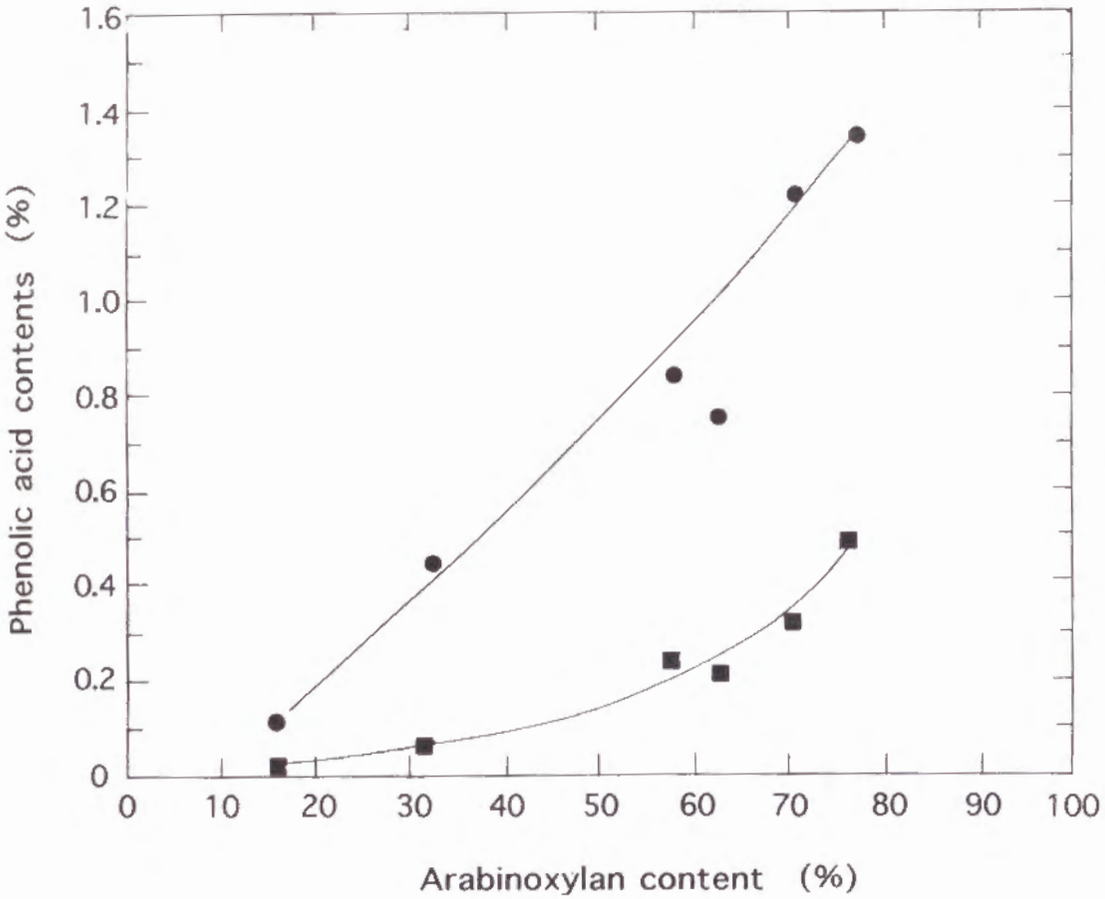

Fig. 4.1 Relationship between phenolic acid and arabinoxylan contents in DMSO-soluble polysaccharides containing phenolic acids.
●, ferulic acid; ■, p-coumaric acid.

the idea that the origin of previously isolated feruloylated arabinoxylo-oligosaccharides from bamboo shoot of Mosochiku is arabinoxylan and that ferulic acid is present in association with arabinoxylan.

4.3.2 Methylation analysis

The results of methylation analysis are summarized in Table 4.2. Partially methylated alditol acetates of arabinose, xylose and glucose could be detected, but those of mannose and galactose were too low to be identified. Major methylated sugars were 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-xylitol derived from 1,4-linked xylopyranosyl residues and 1,4-di-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol derived from 1,4-linked glucopyranosyl residues. The molar % of 1,3,4,5-tetra-*O*-acetyl-2-*O*-methyl-xylitol derived from 3,4-linked xylopyranosyl residue was similar

Table 4.2 Methylation analysis of DMSO-soluble polysaccharides containing phenolic acids
(relative molar %)

Component	Positions of immature bamboo culm					
	No.1	No.2	No.3	No.4	No.5	No.6
2,3-Me2-Ara[a]	0.3	0.3	0.5	0.3	0.2	0.7
2,3,5-Me3-Ara	3.0	3.3	3.5	3.8	3.6	1.3
2-Me-Xyl	3.9	3.7	4.7	3.7	3.2	0.2
2,3-Me2-Xyl	81.9	87.5	56.2	62.4	23.7	14.0
2,3,4-Me3-Xyl	0.9	1.9	1.5	1.6	1.2	1.3
2,3,6-Me3-Glc	8.5	3.0	30.2	25.0	58.1	74.7
2,4,6-Me3-Glc	1.4	-	1.7	2.1	8.1	0.3
2,3,4,6-Me4-Glc	0.1	0.3	1.7	1.1	2.1	0.3

[a] 2,3-Me2-Ara : 1,5-di-*O*-acetyl-2,3-di-*O*-methyl-arabinitol *etc.*

to that of 1,4-*O*-acetyl-2,3,5-tri-*O*-methyl-arabinitol from terminal arabinofuranosyl residue. These results indicate that xylan in the DMSO-

soluble polysaccharide containing phenolic acids has a backbone of 1,4-linked xylose residues having arabinofuranosyl pendants at *O*-3. The fact that most glucoses are linked at *O*-4 further supports that the glucose residues, especially at the upper portions, were originated from α-glucan such as starch. The presence of 1,3-di-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol indicates that a small amount of 1,3-linked glucose residues originated from (1,3 ; 1,4)-glucan may also be present as indicated by Kato *et al.* in bamboo shoot (1982).

4.3.3 UV and IR spectroscopic analyses

UV Spectra of the DMSO-soluble polysaccharides containing phenolic acids are shown in Fig. 4.2. All samples except No.6 had a clear absorption maximum (λmax) at 324 nm ascribable to that of esterified ferulic acid and extinction coefficients increased with lowering the position of the culm. The absorption peak at 315 nm due to esterified *p*-coumaric acid, however, could not be detected, because of heavy overlapping with absorption due to esterified ferulic acid and its low content. Similarly, the DMSO-soluble polysaccharide containing phenolic acids from No.6 did not have clear absorption maximum between 300 and 350 nm reflecting its low phenolic acid content (0.03 %).

IR Spectra of the DMSO-soluble polysaccharides containing phenolic acids from No.1 to No.6 are shown in Fig. 4.3. All samples had several characteristic IR bands at 3,400 cm^{-1} (OH), 2,920 cm^{-1} (CH), 1,740 cm^{-1} (esterified carbonyl) 1,620 cm^{-1} (aliphatic conjugated C=C), and 1,510 cm^{-1} (aromatic C=C). The strength of the esterified carbonyl band became prominent as decrease in height. Based on these results, ferulic acids are concluded to be present as esterified forms in addition to acetic acid.

4.3.4 Molecular weight distribution

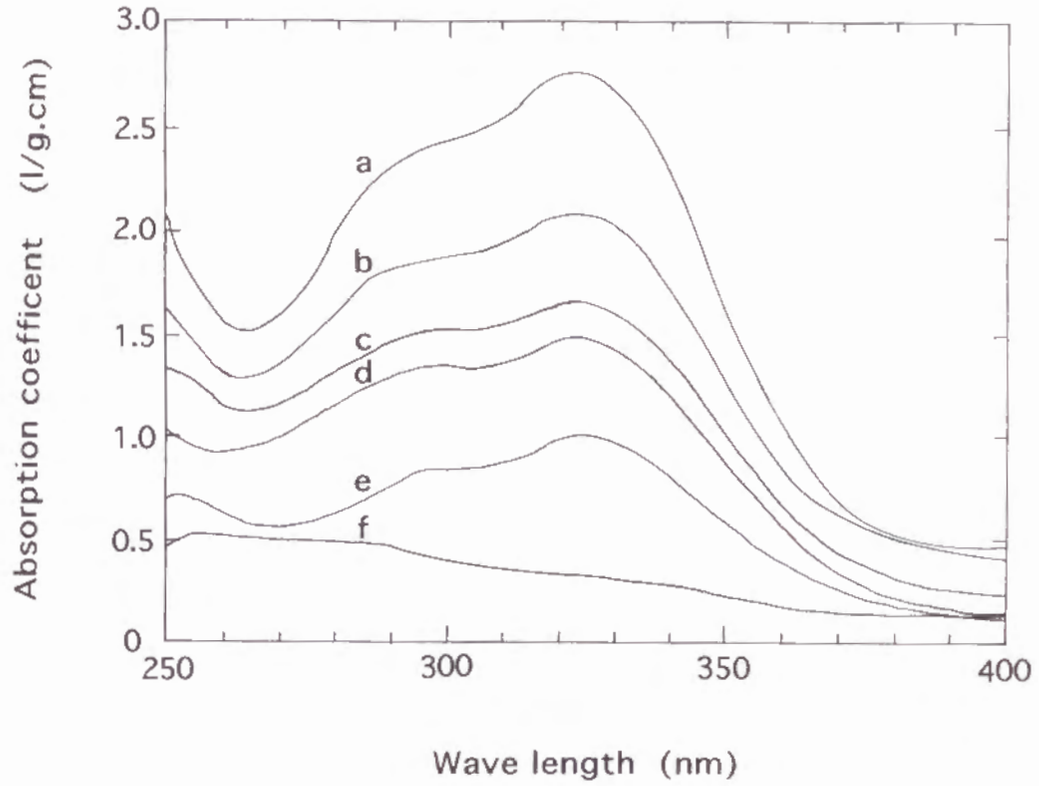

Fig. 4.2 Ultraviolet absorption spectra of DMSO-soluble polysaccharides containing phenolic acids from No.1 to No.6.
(a), No.1; (b), No.2; (c), No.3; (d), No.4; (e), No.5; (f), No.6.

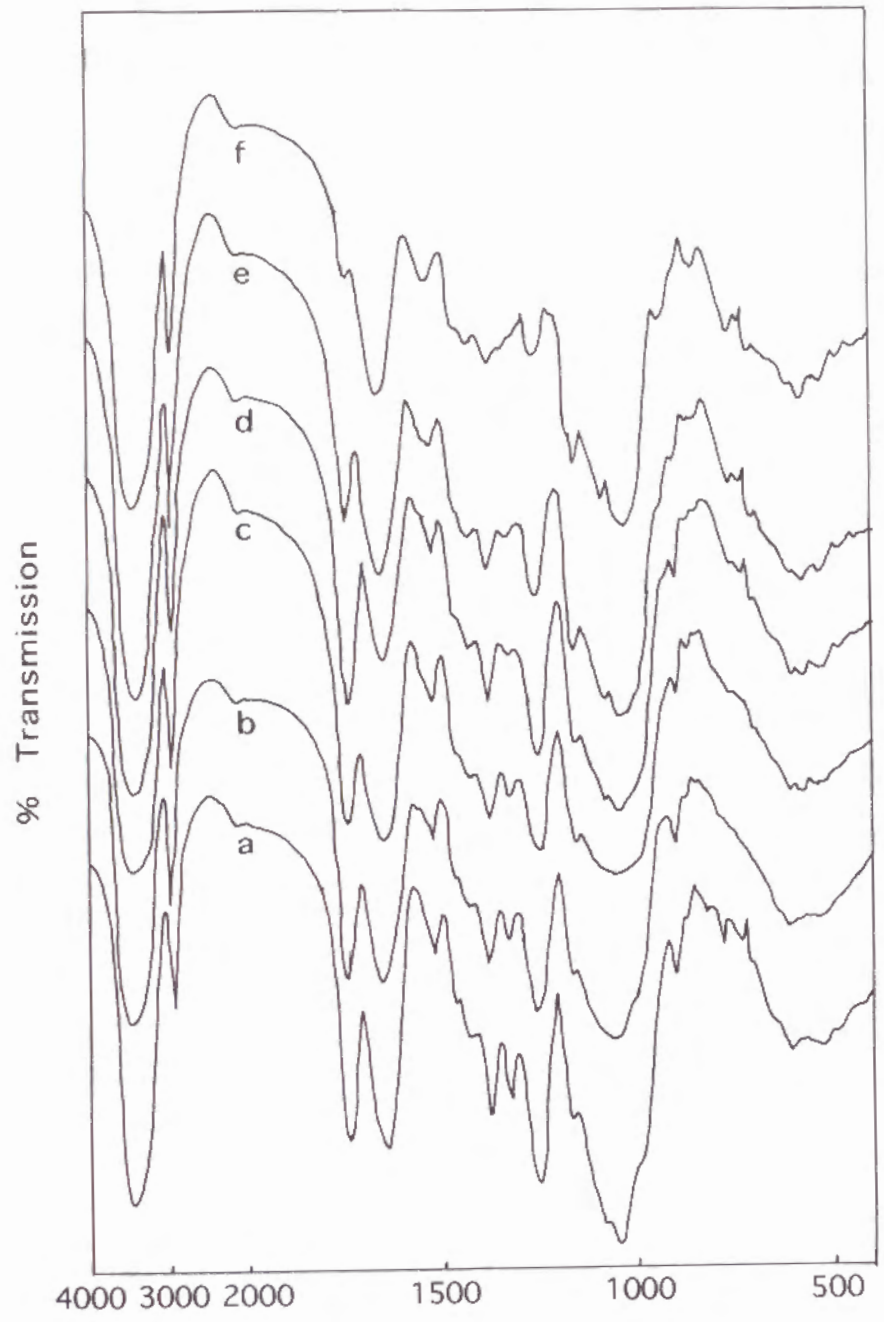

Fig. 4.3 Infrared spectra for DMSO-soluble polysaccharides containing phenolic acids from No.1 to No.6.
(a), No.1; (b), No.2; (c), No.3; (d), No.4; (e), No.5; (f), No.6.

The molecular weight distribution of the DMSO-soluble polysaccharides containing phenolic acids was examined by size exclusion chromatography on Toyo-pearl HW 75 using 80 % aq. DMSO as the eluent. Only the region where samples eluted is shown in Figure 4.4. All elution profiles given from the absorption at 325 nm were similar, but their intensities varied depending upon the content of esterified ferulic acid. The weight average molecular weight ($\overline{Mw}$) at maximum absorption position was estimated to be constant at 1.6×10^5. Some differences were, however, seen in the elution profiles given from the absorption at 480 nm. At No.1, the absorption at 325 nm was the highest and overlapped with that of 480 nm, supporting the feruloylation of the polysaccharide in the samples. The elution profiles from No.2 to No.5 showed shoulders before and/or behind the peak, indicating heterogeneous in size distribution of the carbohydrate portions. In the case of No.6, the position having the maximum absorption at 480 nm was further shifted to the lower molecular region at 1.0×10^5.

These data may suffice to conclude that the molecular weights of the native arabinoxylan esterified with ferulic acid are kept constant throughout the change in maturation from No.6 to No.1.

4.3.5 NMR spectroscopic analysis

For further structural analysis of the DMSO-soluble polysaccharides, their NMR spectra were measured. One dimensional ^{13}C-NMR spectra of the samples from No.1 and No.6 at the ring carbon region are shown in Fig. 4.5. In the case of No.6, six intense signals and several minor signals were seen before glucoamylase treatment. Since the former six signals diminished after glucoamylase treatment and overlapped with those of soluble starch, the DMSO-soluble material from No.6 was concluded to contain a large amount of starch, supporting the chemical compositional and methylation analyses. The starch carbon signals were assigned as 100.3 ppm (C-1), 79.4 ppm (C-4), 73.4 ppm (C-3), 72.0 ppm (C-2), 71.8

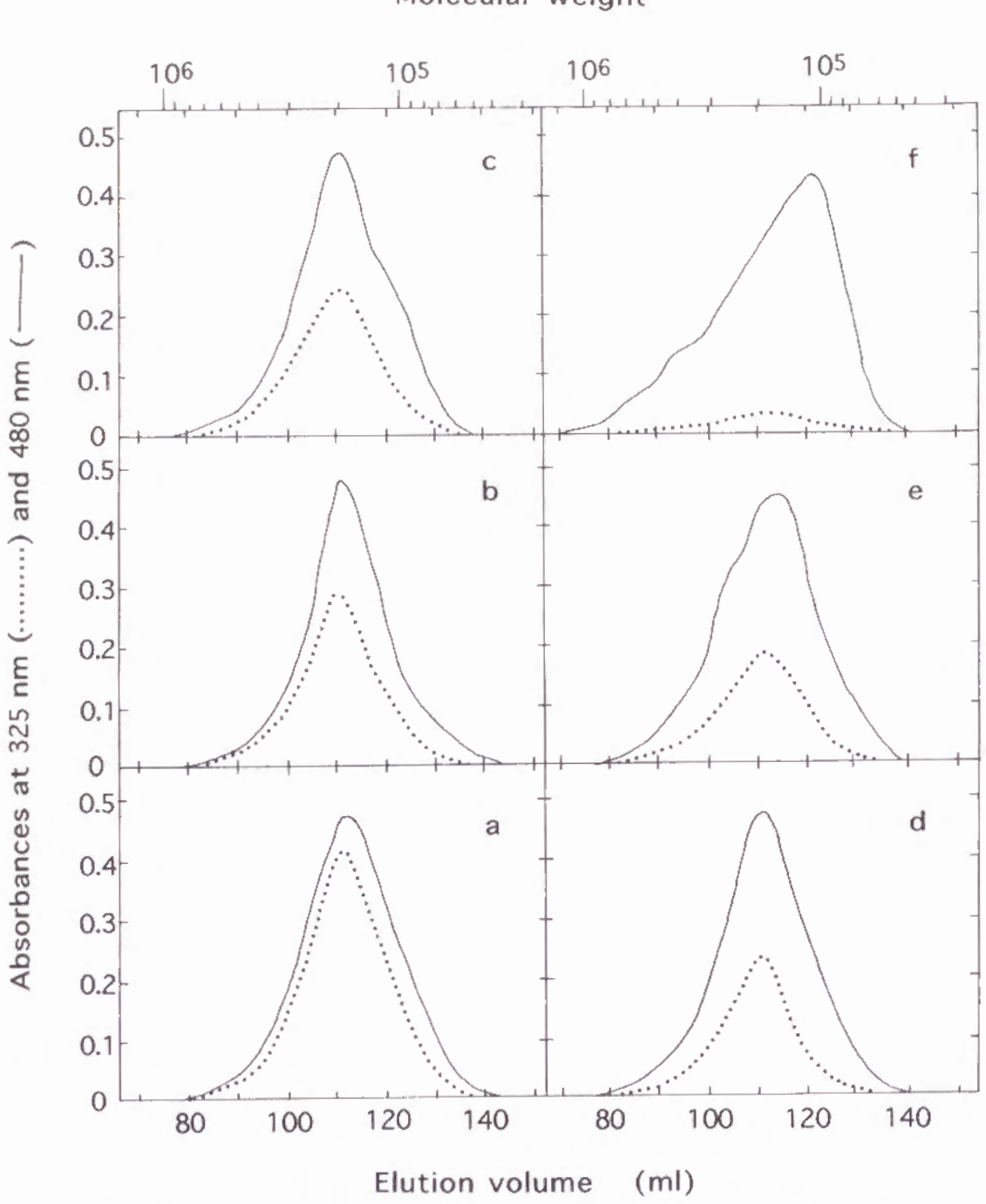

Fig. 4.4 Size exclusion chromatography of DMSO-soluble polysaccharides containing phenolic acids from No.1 to No.6 on Toyo-pearl HW-75.
(a), No.1; (b), No.2; (c), No.3; (d), No.4; (e), No.5; (f), No.6.
The void volume of the colum was situated at 52 ml.
Only the region where sample was eluted is shown in the figure.

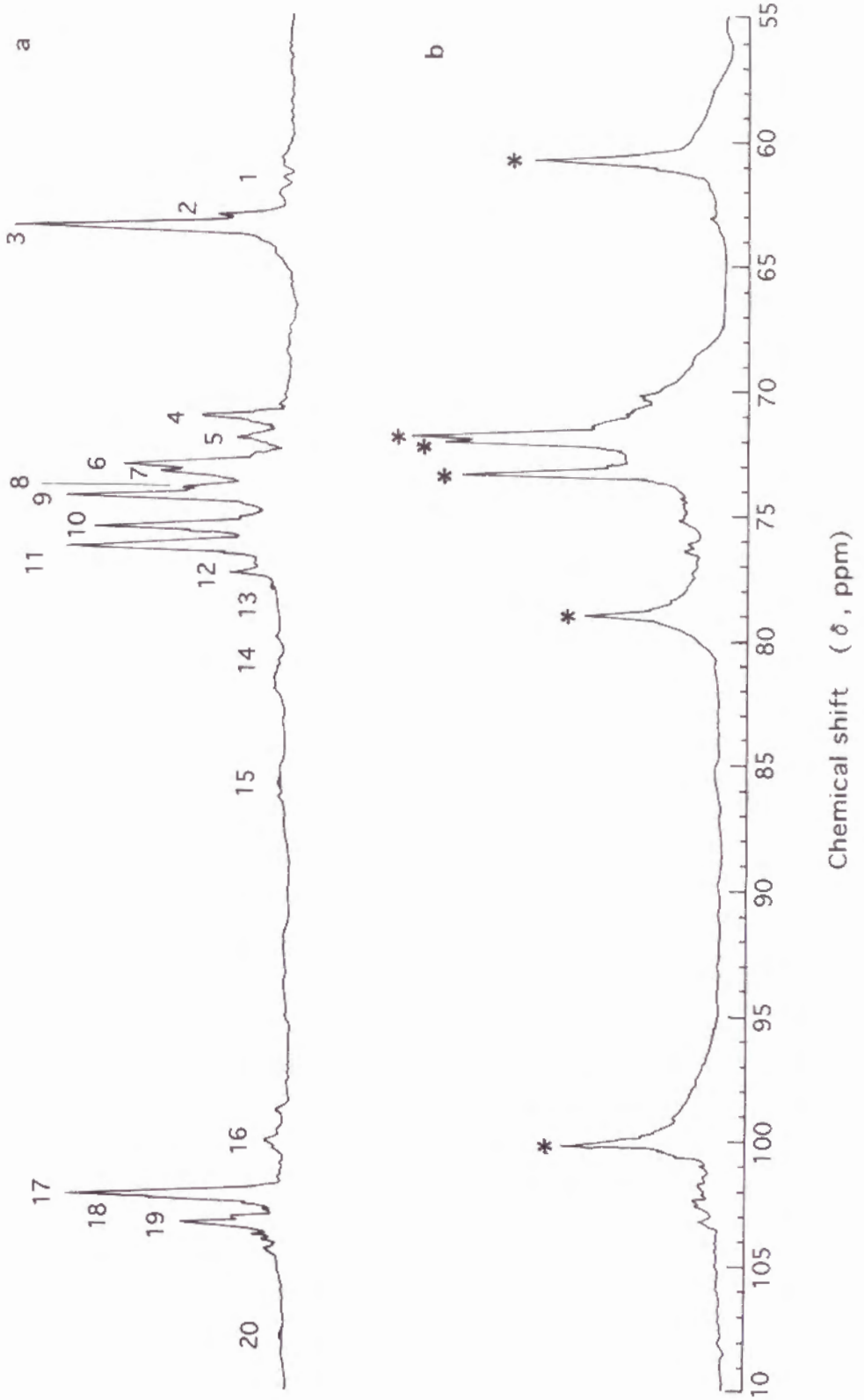

Fig. 4.5 ^{13}C-NMR spectra of DMSO-soluble polysaccharides containing phenolic acids from No.1 (a) and No.6 (b).
The signals shown by asterisks in (b) were due to starch.

Table 4.3 ^{13}C-NMR data for DMSO-soluble polysaccharide containing phenolic acids from No.1

Peak No.[a]	Chemical shift δ (ppm) [b]		Assignment
1	61.94	C-5	α-L-Ara*f*-
2	62.83	C-5	4)-β-D-Xyl*p*(AcO-2)-
3	63.33	C-5	4)-β-D-Xyl*p*-, 4)-β-D-Xyl**p*-(1→4)-β-D-Xyl*p*(AcO-3)-
4	70.87	C-2	4)-β-D-Xyl*p*(AcO-3)-
5	71.81	C-3	4)-β-D-Xyl*p*(AcO-2)-
6	72.80	C-2	4)-β-D-Xyl*p*-
7	73.12	C-2	4)-β-D-Xyl**p*-(1→4)-β-D-Xyl*p*(AcO-3)-
8	73.74	C-2	4)-β-D-Xyl*p*(AcO-2)-
9	74.06	C-3	4)-β-D-Xyl*p*-,
		C-3	4)-β-D-Xyl**p*-(1→4)-β-D-Xyl*p*(AcO-3)-
10	75.26	C-3	4)-β-D-Xyl*p*(AcO-3)-
		C-4	4)-β-D-Xyl*p*(AcO-3)-
		C-4	4)-β-D-Xyl**p*-(1→4)-β-D-Xyl*p*(AcO-3)-
11	76.02	C-4	4)-β-D-Xyl*p*-
12	77.04	C-4	4)-β-D-Xyl*p*(AcO-2)-
13	77.89	C-3	α-L-Ara*f*-
14	80.69	C-2	α-L-Ara*f*-
15	85.87	C-4	α-L-Ara*f*-
16	99.74	C-4	4)-β-D-Xyl*p*(AcO-2)-
17	101.81	C-1	4)-β-D-Xyl*p*(AcO-3)-
18	101.89	C-1	4)-β-D-Xyl*p*-
19	103.01	C-1	4)-β-D-Xyl**p*-(1→4)-β-D-Xyl*p*(AcO-3)-
20	107.48	C-1	α-L-Ara*f*-

[a] Peak No. spedified in Fig. 4.5.
[b] Chemical shifts relative to DMSO-*d*6 (39.50 ppm) in DMSO-*d*6 : D2O (3 : 17, v/v) at 70 °C.

ppm (C-5) and 60.8 ppm (C-6). As the content of starch decreased, the signals due to native partially acetylated arabinoxylan became evident. Unfortunately, however, all ^{13}C-signals due to phenolic acids were hidden in the base line of the spectra because of their low contents. Table 4.3 lists the assignments of the signals (shown in Fig. 4.5).

Figure 4.6 shows one dimensional ^{1}H-NMR spectra of the DMSO-soluble polysaccharide containing phenolic acids from No.1 after glucoamylase treatment. In contrast to ^{13}C-NMR spectra, spectra clearly showed an existence esterified ferulic acid in the region from 6.0 ppm to 8.0 ppm except methoxyl proton which appeared at 3.23 ppm. A weak signal due to H-1 of α-glucan remained after glucoamylase treatment was also detected at 5.06 ppm. Based on the NMR data, the native arabinoxylan present in the DMSO-soluble polysaccharides containing phenolic acids was demonstrated to contain esterified ferulic acid.

4.3.6 Identification of a feruloylated oligosaccharide

In order to further characterize the position of feruloylation, enzymatic digestion of the DMSO-soluble polysaccharide was carried out. Since the material extracted from the lowest portion (No.1) contained the highest amounts of arabinoxylan and ferulic acid, this material was tried to digest with Driselase, a cellulase preparation devoid of feruloyl arabinoxylan esterase. The hydrolysate was subjected to adsorption chromatography of Sephadex LH-20 (Fig. 4.7) and a ferulic acid rich fraction shown as a bar in the figure was collected (yield; 9.6 % of the original DMSO-extract). When this fraction was analyzed by HPLC, UV elution profile indicated the presence of two peaks (I, 12.4 min and II, 13.0 min). Therefore this material was further analyzed by LC/MS. Figure 4.8 shows total ion chromatograph, selective mass-chromatogram at m/z 589, and UV elution profile monitored at 325 nm. The UV elution profiles (Fig. 4.8a) indicate the presence of two peaks (I, 5.9 min and II, 6.5 min) due to

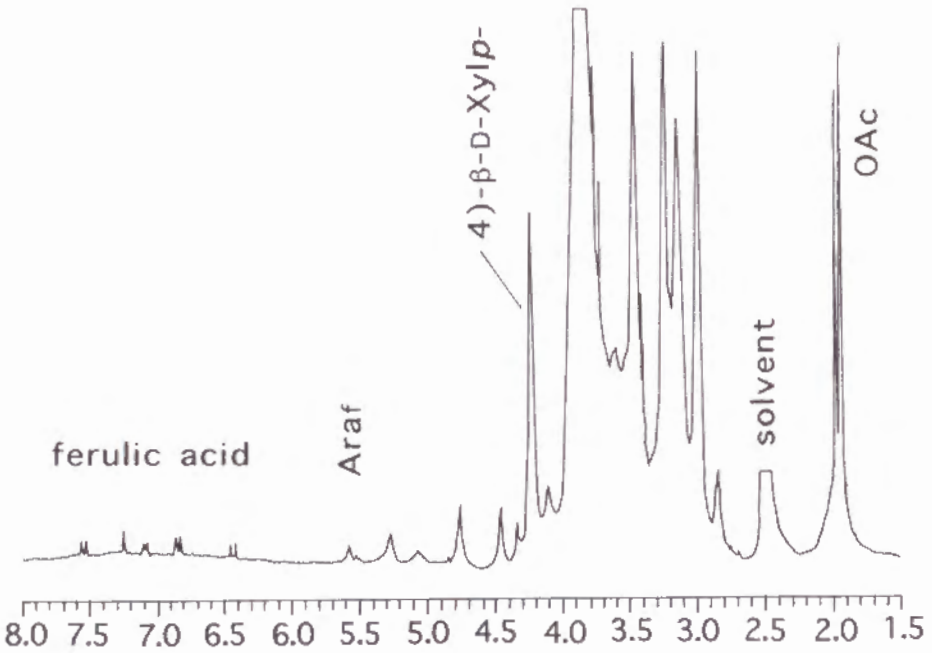

Fig. 4.6 ^{1}H-NMR spectrum of DMSO-soluble polysaccharide containing phenolic acids from No.1 after glucoamylase treatment.

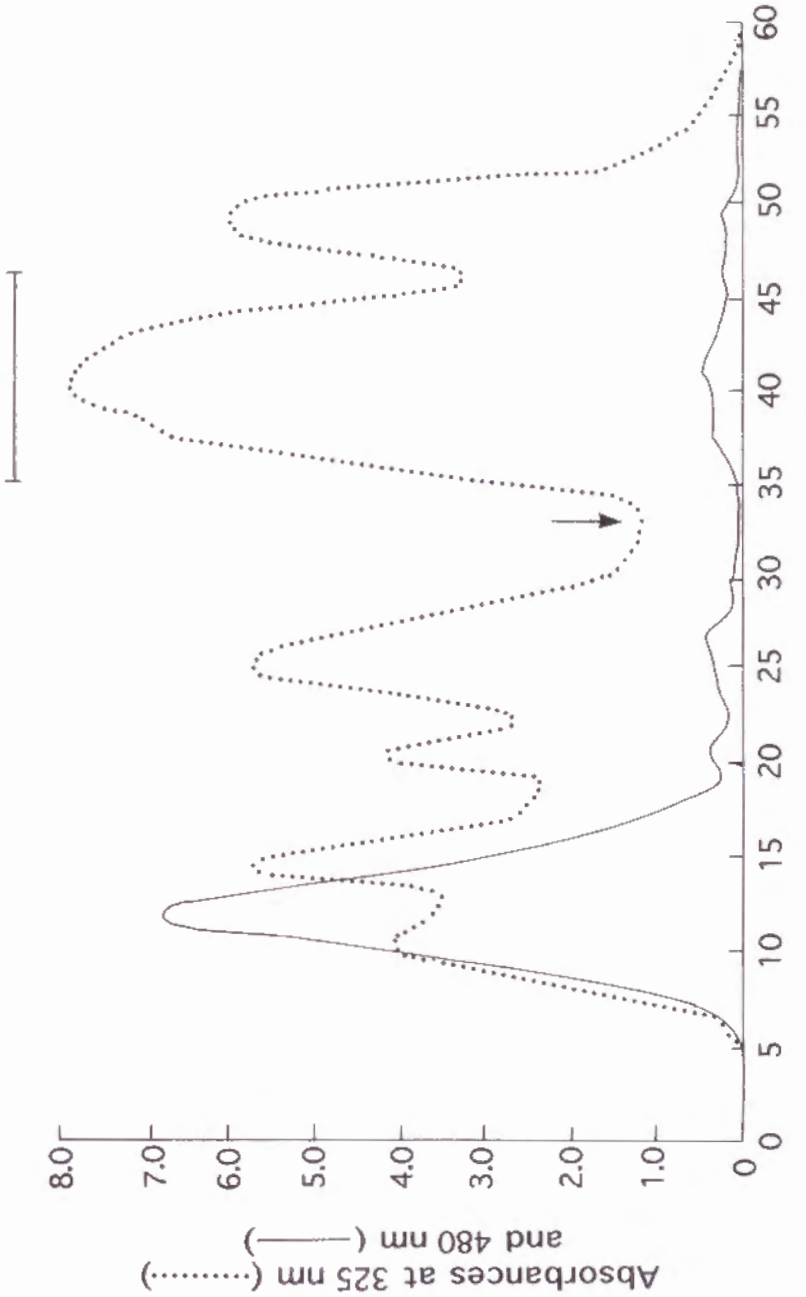

Fig. 4.7 Adsorption chromatography of the enzymatic hydrolysate of DMSO-soluble polysaccharide containing phenolic acids from No.1 on Sephadex LH-20.
An arrow shows the changing position of elution solvent from water to 50 % aq. dioxane.

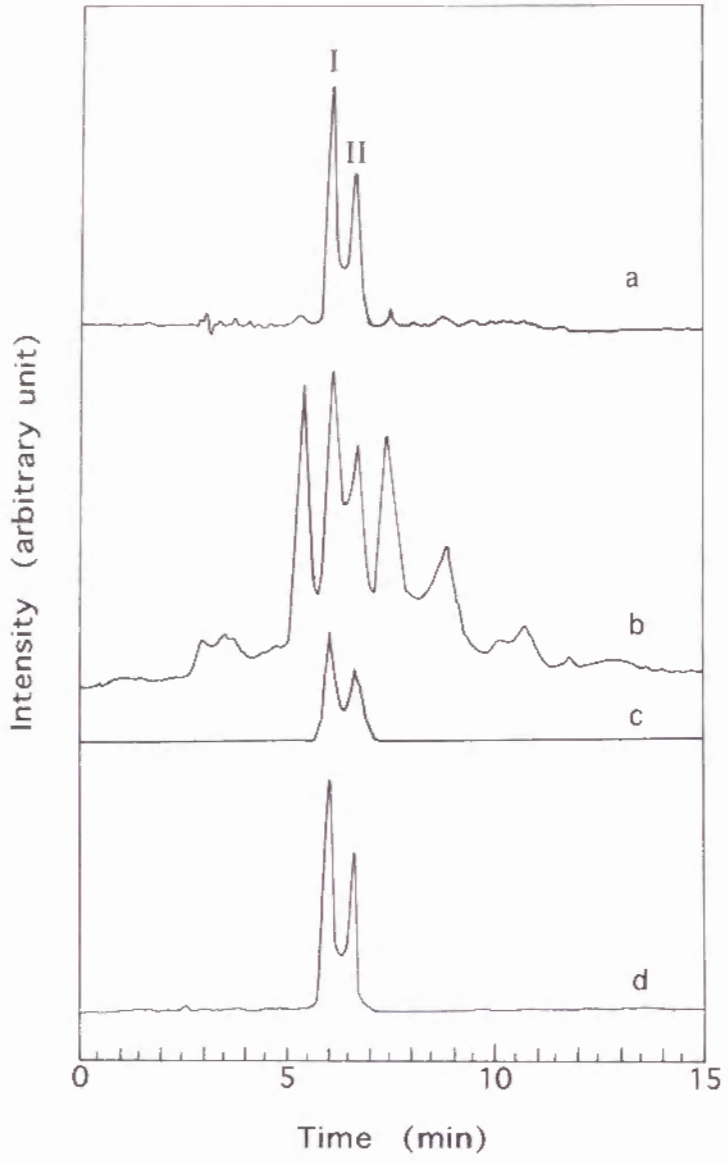

Fig. 4.8 LC/MS chromatogram and HPLC elution profile of feruloylated oligosaccharide fraction from No.1 and the standard feruloylated arabinoxylobiose (FAX2).
(a) : HPLC elution profile of the feruloylated oligosaccharide fraction monitored at 325 nm.
(b) : Total ion chromatogram of the feruloylated oligosaccharide fraction.
(c) : Selective ion chromatogram at m/z 589.
(d) : HPLC elution profile of a standard FAX2 which was resolved into two peaks (I and II) corresponding to β and α anomers.

a feruloylated oligosaccharide. The retention times of there peaks are found to be coincident with the authentic FAX2 (Fig. 4.8d). The negative-ion API mass spectra at these peaks also coincident with the authentic FAX2 and contained an intense ion at m/z 589 corresponding to $(M-H)^+$ quasimolecular ion (Fig. 4.8c) composed of one ferulic acid and three pentose residues. In addition, fragment ions at m/z 457, 325 and 193, generated by loss of one, two and three pentose residues, respectively, were detected. The difference between these two peaks was observed only in the intensities of the fragment ions. Since previous NMR data of the authentic FAX2 showed that the reducing end xylose is present as a tautomeric equilibration mixture of α and β forms in a molar ratio of 2:3 (Kato *et al.*, 1983) which is close to the intensity of these peaks, two peaks appeared on the chromatogram was corresponded to α and β anomers of FAX2. Resolution due to *trans* and *cis* isomers was excluded because rechromatography of the fractions corresponding to each peak gave similar profiles as shown in Fig. 4.8d. The structure of the main arabinoxylan-oligosaccharide was further analyzed by 2D-NMR after isolation and purification by preparative HPLC. The results clearly proved that its primary structure was [*O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose]. The assingment of ^{1}H- and ^{13}C-signals are listed in Table 4.4 and Table 4.5, confirming the results of Kato *et al.* (1983) and Ishii and Hiroi (1991b).

Based on the data presented above, ferulic acid present in the DMSO-extracts were concluded to be esterified with arabinosyl pendants of arabinoxylan. Degree of feruloylation increased with maturation from the top (one ferulic acid per 28 arabinose residue) to the bottom (one ferulic acid per 4 arabinose) of the same immature culm of bamboo. These results present a direct evidence that the native arabinoxylan in the cell wall of Mosochiku was esterified with ferulic acid.

Table 4.4 ^{1}H-NMR data for isolated oligomer

	Xyl*p*		Xyl**p*	Ara*f*	Ferulic acid					
	H-1α	H-1β	H-1	H-1	H-2	H-5	H-6	H-7	H-8	OMe
Chemical shift (δ, ppm)	5.18	4.59	4.47	5.26	7.14	6.86	7.07	7.56	6.34	3.90
J 1,2 (Hz)	3.60		7.4	7.6	1.8	8.00	1.80, 8.00	16.0	16.0	

Table 4.5 ^{13}C-NMR data for isolated oligomer

	Chemical shift (δ, ppm)									
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	OMe
Xyl*p* α	92.9	71.8	72.2	77.5	59.7					
β	97.4	74.8	74.8	77.3	63.9					
Xyl**p*	102.6	73.3	82.4	68.6	65.9					
Ara*f*	109.1	82.0	77.7	82.8	64.5					
Ferulic acid	127.5	111.9	148.4	148.7	114.6	124.2	147.2	116.7	169.8	56.5

4.4 Summary

DMSO-soluble polysaccharides were extracted from six different portions of a 6 m-high immature culm of Mosochiku. Chemical analysis showed that the extracted polysaccharides were mainly composed of partially acetylated arabinoxylan containing a small amount of esterified phenolic acids (ferulic and *p*-coumaric acids) and starch. The contents of arabinoxylan and phenolic acid increased with growth in accordance with lowering the position of the culm, while the reverse relationship was observed in the starch content. The molar ratio of xylose to arabinose increased from the top (5.3) to the bottom (18.9) of the culm. The change of ferulic acid content was in good correlation with that of arabinoxylan content. The position of feruloylation was further demonstrated to be at C-5 of arabinofuranose pendants of arabinoxylan chain by enzymatic hydrolysis and isolation of a known feruloylated arabinoxylobiose, *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose. Degree of feruloylation increased with maturation from the top (one ferulic acid per 28 xylose residues) to the bottom (one ferulic acid per 4 xylose residues) of the same immature culm of Mosochiku. These results present a direct evidence that the native arabinoxylan in the cell wall of bamboo was esterified with ferulic acid.

Chapter 5 Arabinogalactan protein as an indicator of immaturity of Mosochiku culm

5.1 Introduction

Arabinogalactan-protein (AGP) is distributed widely in higher plants. It is a kind of proteoglycan and can be extracted easily with water. In comparison with the proportions of carbohydrate, protein content is low ranging from 2 to 10 %. The main carbohydrate backbone is consisted of a highly branched galactan with arabinofuranose residues as side chains. The most remarkable characteristic of the protein moiety is a high hydroxyproline content (Clarke *et al.*, 1979; Fincher *et al.*, 1983). Many cytochemical (Schopfer, 1990) and immunohistological studies for the distribution of AGP have been done (Clarke *et al.*, 1975, 1978; Pennell *et al.*, 1989; Komalavilas *et al.*, 1991; Kikuchi *et al.*, 1993), and AGP has been shown to be present in middle lamella of cell walls or at plasma membrane. Pennell (1992) reported that the properties of AGPs in middle lamella and at plasma membrane were different and suggested that they played different role in the tissue. According to the function of AGP, although various physiological functions such as recognition of cells and small ligands, water retention and cell-cell adhesion have been suggested, none of these functions have been proved. Recently Yasufuku *et al.* (1994) reported the importance of AGP in growth of cabbage. Tsumuraya *et al.* (1988) isolated AGPs from primary and mature roots of radish and found some differences in their structures. These results are interesting for considering the participation of AGP in cell growth.

Previous studies on AGP were mainly concerned with dicotyledon and information about monocot AGP was scarce (Pollard and Fincher, 1981; Mascara and Fincher, 1982). In the previous Chapters 1 and 2, the

presence of arabinogalactan or related polysaccharide in the immature portion of Mosochiku has been pointed out.

In this chapter, AGP was isolated from six different portions of a 6 m-high culm of Mosochiku and their chemical properties were characterized in relation to its maturity. In addition, since the previous study on the cabbage AGP showed that the water-soluble AGP was transformed into insoluble forms with leaf hardening (Yasufuku *et al.*, 1987), the author tried to isolate a new type AGP from the residue remained after water extraction by further extraction with DMSO followed by aq. calcium chloride solution.

5.2 Materials and methods

5.2.1 Preparation and purification of AGPs

An immature Mosochiku having 6 m in height was harvested on May in 1992 at the Botanical Garden of Kyoto University and divided into 6 portions having 1 m in height, No.1 to No.6, from the bottom to the top as described in 1.2.1. Each portion was lyophilized and milled to pass 24 mesh screen by a Wiley mill. Each bamboo powder was extracted with 75 % aq. ethanol and water. The water extract was poured into 3 vol. of ethanol. The precipitate was filtrated, and washed with methanol and acetone, and dried up to get water-soluble material. The water-soluble material was dissolved in 0.1 M borate buffer (pH 9.0) and subjected to gel filtration chromatography on a column of Sepharose CL-4B (3.5 x 52.5 cm) equilibrated with the same buffer. Elution was also made with the same buffer. A portion was taken out from each fraction and the color was developed by the method of phenol-sulfuric acid for detection of carbohydrate (Dubois *et al.*, 1956). Absorbance at 280 nm was monitored for detection of protein. Higher molecular weight fractions were gathered, dialyzed against water and lyophilized. This sample was solubilized in 50

mM citrate buffer (pH 3.0) and filtered through a column (1.2 x 8.0 cm) of SP-Toyopearl 650M using the same buffer as an eluent. The unadsorpted material was further subjected to anion exchange chromatography on a column of DEAE-Toyopearl (1.7 x 13.5 cm). The elution solvent was 25 mM sodium phosphate buffer (pH 6.9) and adsorbed materials were eluted with a linear gradient (0-1 M) of sodium chloride in the same buffer.

The residue from No.4 obtained after extraction with water was depectinated with 0.25 M CDTA (1,2-cyclohexanediamine tetraacetic acid) and extracted with DMSO for 24 hr as described in 4.2.1. The residue was further extracted with a 0.2 M aq. calcium chloride solution. The extract was dialyzed and lyophilized to give a salt-soluble material. This material was applied on the same column of Sepharose CL-4B with 50 mM sodium phosphate buffer (pH 6.8) as an eluent and further subjected to cation and anion exchange chromatographic separations SP- and DEAE-Toyopearl as described above. All chromatographic operations were carried out at room temperature.

5.2.2 Chemical analyses of AGP

The uronic acid content was determined by the methods of Filisetti-Cozzi and Carpita (1991). The total sugar content was determined by the method described in 2.2.2. The neutral sugar composition was analyzed as described in 1.2.4. Methylation analysis was carried out as described in 4.2. by using a column of DB-225 (0.25 mm x 15 m). The column temperature was increased from 140 °C to 220 °C at a rate of 2 °C / min and maintained at this temperature for 15 min. The amino acid composition was analyzed by a Hitachi 835 amino acid analyzer after hydrolyzation with 6 N hydrochloric acid at 110 °C for 24 hr. The protein content was calculated by summing up the weight of amino acids.

5.2.3 Measurement of molecular weight

Molecular weight of the purified AGP was determined by gel permeation chromatography (GPC) on a column of YMC-diol S-5 200A (7.5 mm x 50 cm) eluted with 50 mM sodium phosphate buffer (pH 6.8) containing 0.02 % sodium azide at 60 °C at a flow rate of 1.0 ml/min. Elution was monitored by Shodex RI SE-51 differential refractometer. The pullulans having known molecular weights were used as a standard as described in 4.2.4.

5.2.4 Polyacrylamide gel electrophoresis

Slab gel electrophoresis was carried out in 7.5 % polyacrylamide gel as described by Ornstein and Davis (1964) in Tris-glycine buffer (pH 8.9) at a current of 12 mA. The gel was stained for protein with 0.025 % Commassie Brilliant Blue R-250, for carbohydrate with the periodic acid-Shiff (PAS) reagent following the procedure of Fairbanks *et al.* (1971) after fixing the gel with 10 % aq. acetic acid solution containing 25 % 2-propanol. The eluting position of AGP was determined by staining with β-galactosyl Yariv antigen synthesized by the method of Yariv *et al.* (1962) at 40 °C for 15 min. The gel plate was dried after washing out the unstained antigen with 0.15 N aq. sodium chloride solution (Yariv *et al.*, 1967).

5.2.5 Agar-gel diffusion

Agar-gel diffusion was carried out according to Ochterlony (1958) using 1 % (w/v) agarose in 0.05 M veronal buffer (pH 8.35). In the central well a solution of AGP from No.6 was inserted, while α-, and β- galactosyl Yariv antigen and β-glucosyl Yariv antigen were filled in surrounding wells.

5.2.6 Spectroscopic analysis

^{13}C-NMR spectra of AGP were recorded with a Brucker ARX-500 spectrometer in D_2O as described in 4.2.7.

5.3 Results and discussion

5.3.1 Purification of water-soluble AGP

Water-soluble materials extracted from six different portions (No.1 to No.6) of the same culm of immature Mosochiku were separated from lower-molecular weight contaminants by elution through Sepharose CL-4B gel. Figure 5.1 shows an elution profile of water-soluble material from No.4. The elution profiles of the other portions (No.1-3, 5, 6) were similar to that of No.4. As AGP has high carbohydrate content, the fractions having high absorptions at 480 nm (317 ml to 413 ml) were gathered, passed through SP-Toyopearl and subjected to anion exchange chromatography on DEAE-Toyopearl. Figure 5.2 shows an elution profile of the partially purified AGP from No.4 on DEAE-Toyopearl. By gradient elution with sodium chloride, adsorbed fractions (108 ml-128 ml) having both carbohydrate and protein were eluted at 0.2 M sodium chloride and could be separated from contaminating materials. This fraction was dialyzed and lyophilized to give water-soluble AGP.

5.3.2 Polyacrylamide gel electrophoresis

Gel electrophretic patterns of water-soluble AGPs are shown in Fig. 5.3. Each purified AGP from No.1 to No.6 showed one broad red band having similar mobility when stained with β-Yariv antigen. When the gels were stained with Coomassie Brilliant Blue, only a very weak blue band was detected at the same position that is stainable with Yariv antigen (data not shown), supporting that the purification of AGP was completed.

5.3.3 Precipitation reaction by gel diffusion

A representative gel diffusion pattern of water-soluble AGP from No.6 is shown in Fig. 5.4. The precipitation lines were formed selectively around the wells between AGP and β-glycosyl Yariv antigens. The stainability of the isolated AGP with β-glycosyl antigens indicates that

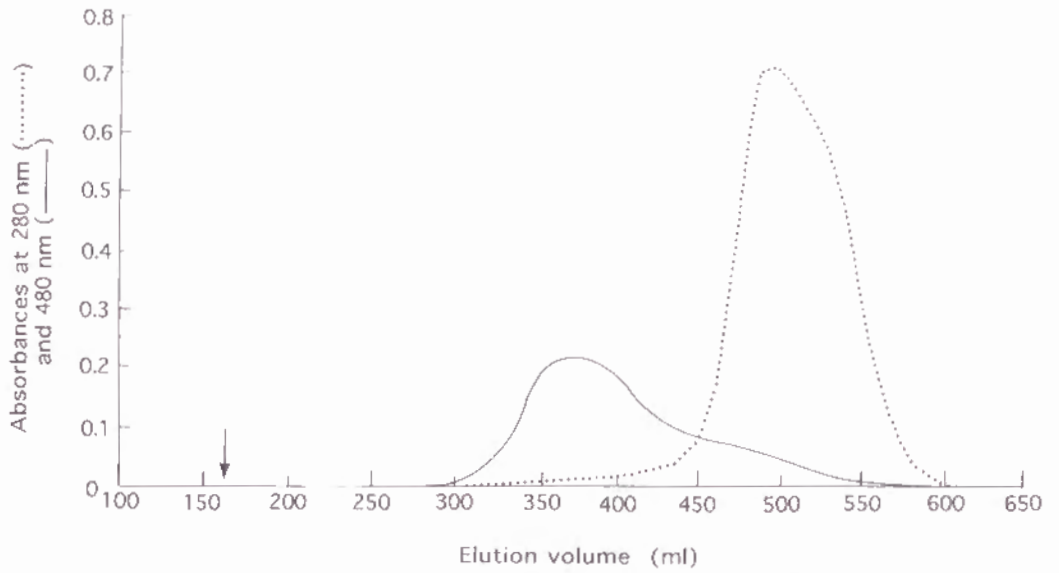

Fig. 5.1 Gel filtration of water-soluble material from No.4 on Sepharose CL-4B. An arrow represents the void volume of the culm.

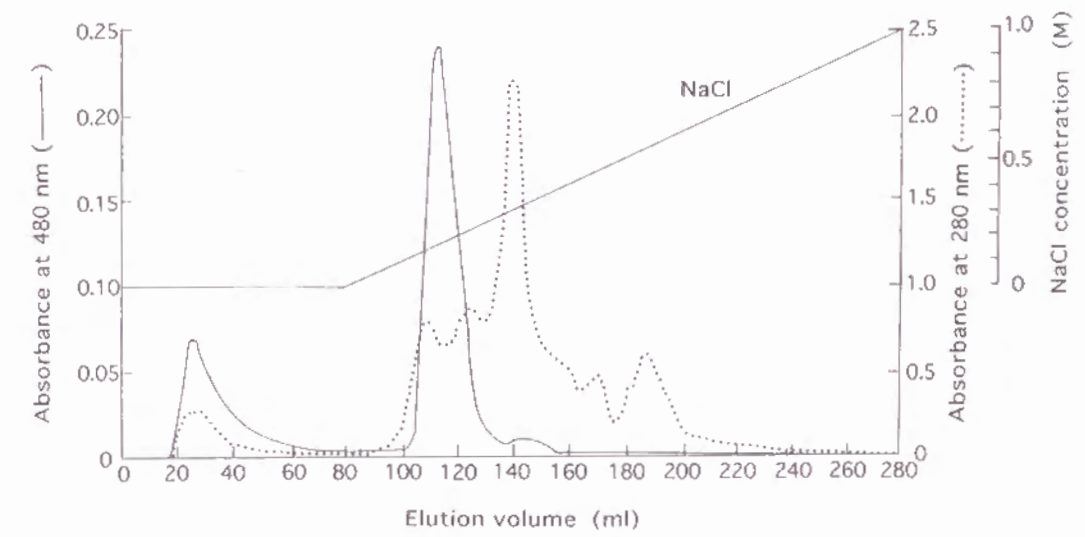

Fig. 5.2 Elution profile for purification of water-soluble arabinogalactan protein from No.4 on DEAE-Toyopearl.

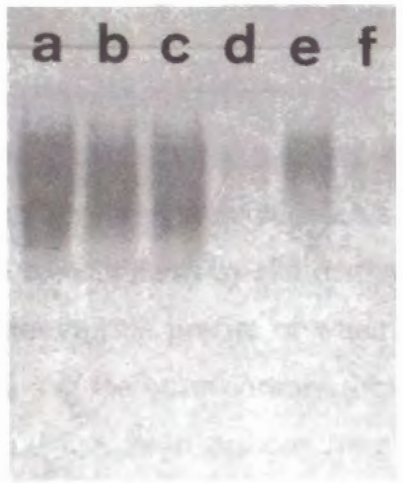

Fig.5.3 Electrophoretic patterns of purified water-soluble arabinogalactan proteins from No.1 to No.6 stained with β-galactosyl Yariv antigen.
(a), No.1; (b), No.2; (c), No.3; (d), No.4; (e), No.5; (f), No.6.

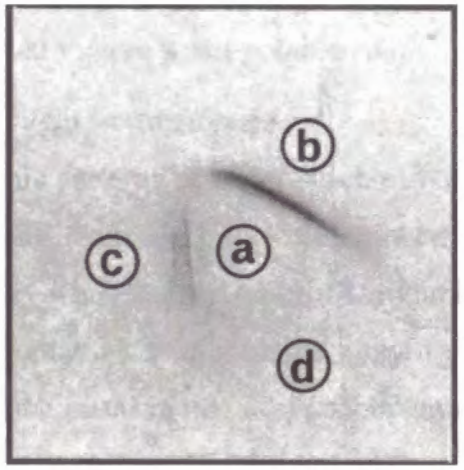

Fig. 5.4 Gel diffusion pattern of water-soluble arabinogalactan protein from No.6 with α- and β-galactosyl and β-glucosyl Yariv antigen.
Wells : (a), arabinogalactan protein from No.6;
(b), β-galactosyl Yariv antigen;
(c), β-glucosyl Yariv antigen;
(d), α-galactosyl Yariv antigen.

bamboo AGPs have a β-lectin-like property as observed in the dicot AGPs (Fincher *et al.*, 1983).

5.3.4 Chemical properties of AGPs

Chemical properties of the isolated water-soluble AGPs are summarized in Table 5.1. Yield of AGP was found to decrease with maturation : about 0.1 % at upper portion (No.5 and 6), while 0.009 % at lower portion (No.1). Since the secondary cell walls became thicker than the plasma membrane and the primary cell wall with progress of maturation, the proportion of the latter two parts in the whole weight of the sample became lower as lowering the position of the culm, supporting the previous results that AGP was distributed in middle lamella and at plasma membrane (Pennell, 1992). The present results also suggest that the water-soluble AGP is not distributed in the secondary cell walls. The weight average molecular weights ($\overline{Mw}$) of the isolated AGPs were constsant at about 50kDa. The protein and uronic acid contents were almost always about 10 % and 7 %, respectively. Carbohydrate was always consisted of L-arabinose and D-galactose, whose ratio was nearly equal to unity.

Table 5.1 Yields and properties of water-soluble arabinogalactan proteins

	Positions of immature bamboo culm					
	No.1	No.2	No.3	No.4	No.5	No.6
Yiled ($x10^{-2}$ %)	0.9	1.8	3.4	3.6	10.0	13.3
Molecular weight (kDa)	51	45	50	50	53	53
Carbohydrate content (%)	82.0	85.5	81.1	82.6	82.4	80.1
Uronic acid content (%)	6.8	6.6	7.9	6.9	7.2	7.6
Protein content (%)	11.9	7.9	11.0	10.5	10.5	12.4
Neutral sugar composition (relative weight %)						
L-Arabinose	48.2	49.6	51.1	49.9	53.6	47.3
D-Galactose	50.0	50.4	48.4	49.5	46.0	52.6
D-Glucose	1.8	-	0.5	0.6	0.4	-

The result of methylation analysis is shown in Table 5.2. Two and four kinds of methylated sugars originated from arabinose and galactose, respectively, were detected. Two-thirds of arabinose were detected as 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-arabinitol derived from terminal arabinofuranose residues and the rest was 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-arabinitol derived from 1,5-linked arabinofuranose residues. As for galactose residues, the main component was detected as 1,3,6-tri-*O*-acetyl-2,4-di-*O*-methyl-galactitol derived from galactopyranose residues doubly linked at *O*-3 and *O*-6 positions. The amount of 1,3-linked galactopyranose residue was estimated to be higher than that of the 1,6-linked residues because the amount of 1,3-di-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol was higher than that of 1,6-di-*O*-acetyl-2,3,4-tri-*O*-methyl-galactitol. These results indicate that the carbohydrate moiety of AGP in an immature bamboo had (1,3 : 1,6)-galactan having 1,5-linked arabino-oligosaccharides or arabinose as side chains.

Table 5.2 Methylation analysis of arabinogalactan proteins
(relative molar %)

Component	Positions of immature bamboo culm					
	No.1	No.2	No.3	No.4	No.5	No.6
2,3,5-Me3-Ara[a]	30.2	31.7	31.4	35.9	36.6	31.5
2.3-Me2-Ara	18.0	17.9	19.7	14.0	20.0	15.8
2,3,4,6-Me4-Gal	3.6	4.4	3.8	8.3	4.1	2.7
2,4,6-Me3-Gal	3.4	5.7	6.2	12.5	5.7	8.1
2,3,4-Me3-Gal	2.4	2.9	5.0	6.8	7.4	6.3
2,4-Me2-Gal	35.7	37.4	33.4	21.9	27.0	35.5

[a] 2,3,5-Me3-Ara : 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-arabinitol *etc.*

Amino acid compositions of water-soluble AGPs are listed in Table 5.3. The content of hydroxyproline, a characteristic amino acid in AGP, was as high as about 40 % in all portions. L-Alanine, L-threonine and L-serine were also abundant and the sum of these four amino acids occupied more than 80 % of the total amino acids.

Table 5.3 Amino acid composition of arabinogalactan proteins
(relative molar %)

Component	Positions of immature bamboo culm					
	No.1	No.2	No.3	No.4	No.5	No.6
Hyp	45.0	43.4	40.1	43.4	41.0	45.3
Asp	2.4	2.1	2.8	2.7	2.5	2.5
Thr	10.9	11.3	11.2	10.5	9.8	11.1
Ser	8.4	9.5	9.2	8.6	9.0	8.3
Glu	3.5	2.5	3.5	3.6	3.5	3.2
Pro	2.0	2.7	2.5	2.3	2.3	2.1
Gly	2.7	1.9	2.7	2.6	3.0	2.1
Ala	18.6	19.7	20.5	19.4	20.7	19.1
Cys	0.5	0.4	0.6	0.5	0.6	0.6
Val	2.3	2.5	2.5	1.9	1.9	1.7
Met	-	0.1	0.4	0.3	0.4	0.4
Ile	0.2	0.3	0.2	0.2	0.3	0.2
Leu	1.3	1.4	1.7	1.5	1.7	1.3
Tyr	0.2	0.2	0.2	0.2	0.2	0.1
Phe	0.2	0.2	0.3	0.2	0.3	0.2
Lys	1.6	1.7	1.6	1.8	2.4	1.7
His	0.2	0.1	0.1	0.1	0.2	0.1
Arg	+	+	0.1	0.1	0.3	0.1

5.3.5 ^{13}C-NMR spectroscopic analysis of AGPs

As a representative result, ^{13}C-NMR spectrum of AGP from No.6 is shown in Fig. 5.5. The peaks were assigned on the basis of previous data (Karacsonyi *et al.*, 1984; Yasufuku *et al.*, 1985; Cartier *et al.*, 1987;

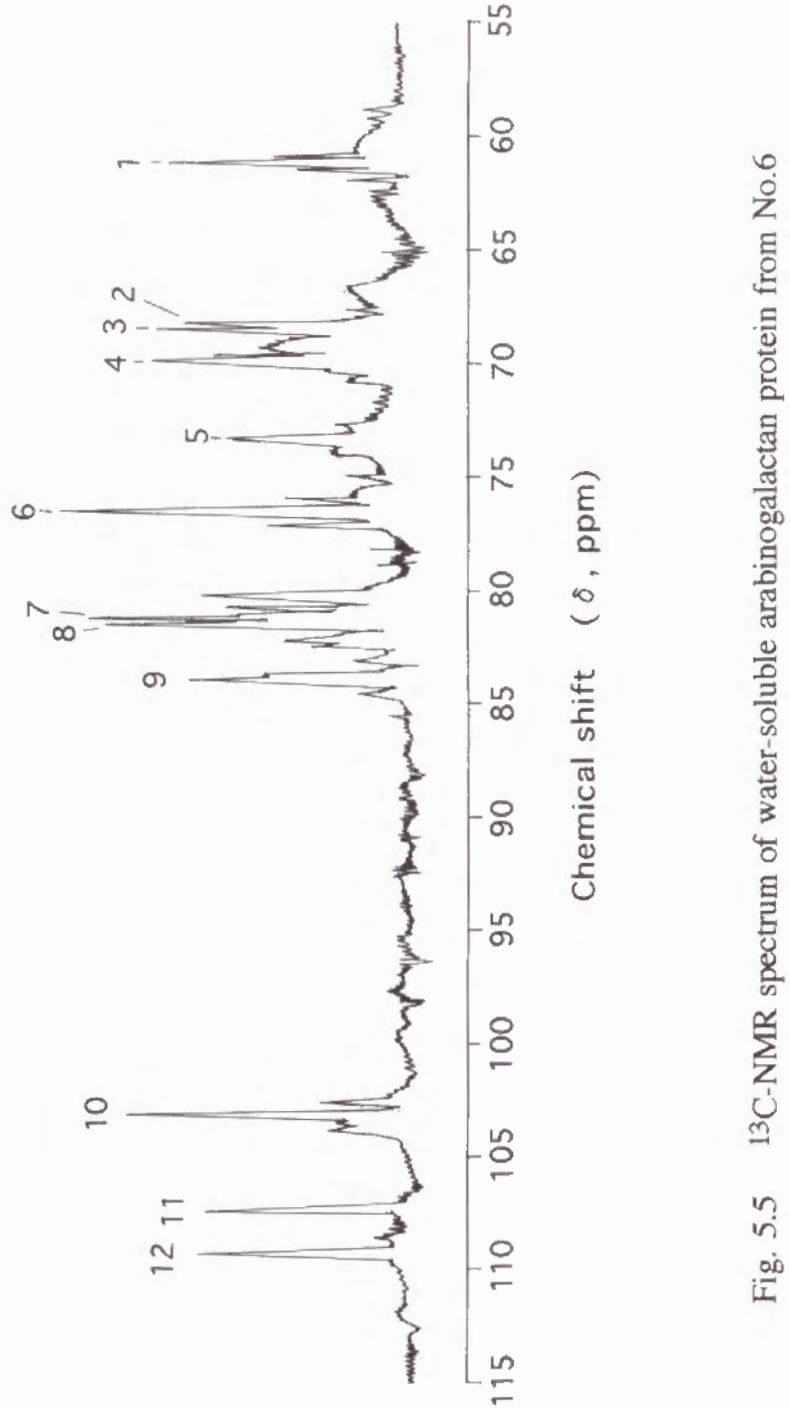

Fig. 5.5 ^{13}C-NMR spectrum of water-soluble arabinogalactan protein from No.6

Table 5.4 ^{13}C-NMR data for water-soluble arabinogalactan protein from No.6

Peak No.[a]	Chemical shift δ (ppm)	Assignment	
1	61.2	C-5	5)-α-L-Ara*f*-
2	68.2	C-6	3,6)-β-D-Gal*p*-
3	68.6	C-4	3,6)-β-D-Gal*p*-
4	70.0	C-2	3,6)-β-D-Gal*p*-
5	73.3	C-5	3,6)-β-D-Gal*p*-
6	76.5	C-3	5)-α-L-Ara*f*-
7	81.0	C-3	3,6)-β-D-Gal*p*-
8	81.5	C-2	5)-α-L-Ara*f*-
9	84.1	C-4	5)-α-L-Ara*f*-
10	103.1	C-1	3,6)-β-D-Gal*p*-
11	107.4	C-1	5)-α-L-Ara*f*-
12	109.3	C-1	α-L-Ara*f*-

[a] Peak No. specified in Fig. 5.5.

Saulnier and Brillouet, 1988) and listed in Table 5.4. In the anomeric carbon region, four signals were observed. The signal at 103.1 ppm was assigned as C-1 of 3,6-di-*O*-substituted galactopyranosyl residue. Two signals at 109.3 ppm and 107.4 ppm were assigned as C-1 of α-L-arabinofuranosyl and 5-*O*-substituted α-L-arabinofuranosyl residue. The last one signal may be assigned. as C-1 from 3-*O* and/or 6-*O* substituted galactopyranosyl residues. Based on the data presented above, it was concluded that the content of water-soluble AGP was closely related to the immaturity of the bamboo and all AGP from different portions of the same culm of an immature bamboo have a common (1,3 : 1,6)-galactan backbone in their carbohydrate moieties.

5.3.6 Purification of salt-soluble AGP

Since Yasufuku *et al.* (1987) showed that the water-soluble cabbage AGP was transformed into water-insoluble forms with leaf hardening, the author finally intended to isolate such a kind of AGP by extracting with aq. calcium chloride solution from the residue given after water extraction as described above. As described in Chapter 1, the sample of No.4 corresponding to 3 to 4 m from the bottom is considered to be the position where the elongation growth almost completed. Gel filtration pattern of the salt-soluble material on Sepharose CL-4B in shown in Fig. 5.6. The peak due to protein existed in lower molecular region as observed in the water-soluble material, but the carbohydrate distribution showed two clear peaks. Two fractions, I (348 ml to 400 ml) and II (420 ml to 480 ml), were individually gathered and their hydroxyproline content was determined. The results indicate that the former fraction I contained about 0.5 % (w/w) of hydroxyproline, while no hydroxyproline could be detected in the latter fraction II. Therefore, only the fraction I was applied on a column of DEAE-Toyopearl. The elution profile is shown in Fig. 5.7. Two adsorbed fractions were obtained as in the case of water-soluble materials (Fig. 5.2). The fractions eluted at salt concentration of 0.2 M were pooled, dialyzed, lyophilized to give salt-soluble AGP.

5.3.7 Characterization of salt-soluble AGP

The yield and chemical properties of the salt-soluble AGP were shown in Table 5.5. The yield of the salt-soluble AGP was very low amounting about one-tenth of the water-soluble AGP. Although molecular weights of both AGP estimated by GPC were almost equal, chemical properties of the salt-soluble AGP were different from those of the water-soluble AGP. In comparison with the water-soluble AGP, protein content increased by 30 % and carbohydrate content decreased by 66 % in the salt-soluble AGP. The ratio of arabinose to galactose decreased from 1 to 0.5. The result of methylation analysis (Table 5.6) indicate that the linkage

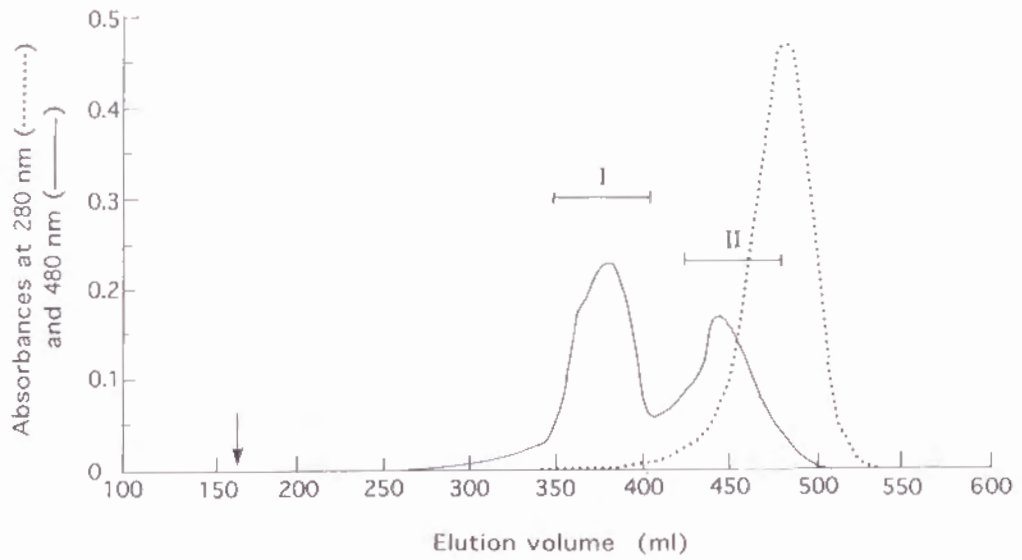

Fig. 5.6 Gel filtration of salt-soluble material from No.4 on Sepharose CL-4B. An arrow represents the void volume of the culm.

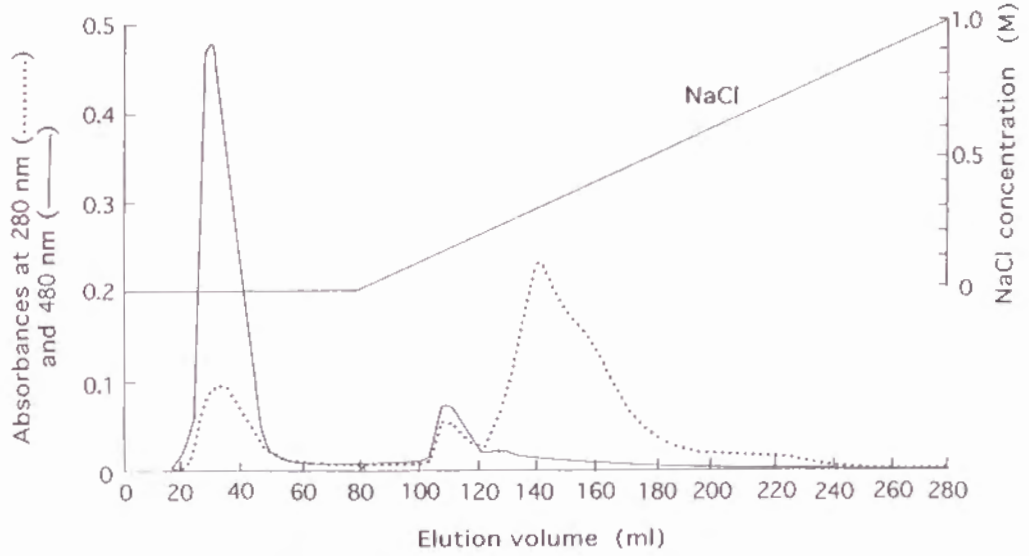

Fig. 5.7 Elution profile for purification of salt-soluble arabinogalactan protein from No.4 on DEAE-Toyopearl.

structure of the carbohydrate moiety of the salt-soluble AGP was similar to that of the water-soluble AGP. Most arabinose residues were located at terminal and 1,3-linked galactan chain was highly branched at *O*-6.

Table 5.5 Properties of salt-soluble arabinogalactan protein from No.4

Yiled (x10^{-2} %)	0.41
Molecular weight (kDa)	49
Carbohydrate content (%)	66.1
Uronic acid content (%)	4.2
Protein content (%)	29.6
Neutral sugar composition (relative weight %)	
L-Arabinose	34.0
D-Xylose	2.1
D-Galactose	59.6
D-Glucose	6.4

Table 5.6 Methylation analysis of salt-soluble arabinogalactan protein from No.4 (relative molar %)

Component	
2,3,5-Me3-Ara[a]	24.6
2,3-Me2-Ara	9.4
2,3,4,6-Me4-Gal	8.4
2,4,6-Me3-Gal	17.9
2,3,4-Me3-Gal	5.0
2.4-Me2-Gal	28.3
2,3,4-Me3-Xyl	2.0
2,3,4,6-Me4-Glc	0.4
2,3-Me2-Glc	2.5
2,4-Me2-Glc	0.4

[a] 2,3,5-Me3-Ara : 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-arabinitol *etc.*

As for amino acid composition (Table 5.7), however, the contents of hydroxyproline, threonine, serine and alanine were high (the sum of these four amino acid being 60 %), but much less than that of water-soluble AGP (80 %).

Table 5.7 Amino acid composition of salt-soluble arabinogalactan protein from No.4 (relative molar %)

Component	
Hyp	31.6
Asp	4.7
Thr	8.2
Ser	7.6
Glu	5.1
Pro	4.3
Gly	6.6
Ala	16.1
Cys	0.9
Val	2.9
Met	0.7
Ile	0.9
Leu	3.0
Tyr	1.1
Phe	1.2
Lys	3.2
His	0.4
Arg	1.5

Electrophoretic patterns of the salt-soluble AGP together with the water-soluble AGP from No.4 are shown in Fig 5.8. These two AGP could not be distinguished by staining with β-galactosyl Yariv antigen, Coomassie Brilliant Blue and PAS reagent.

From these results, both salt-soluble and water-soluble AGPs were concluded to have similar carbohydrate moiety, but differed in protein

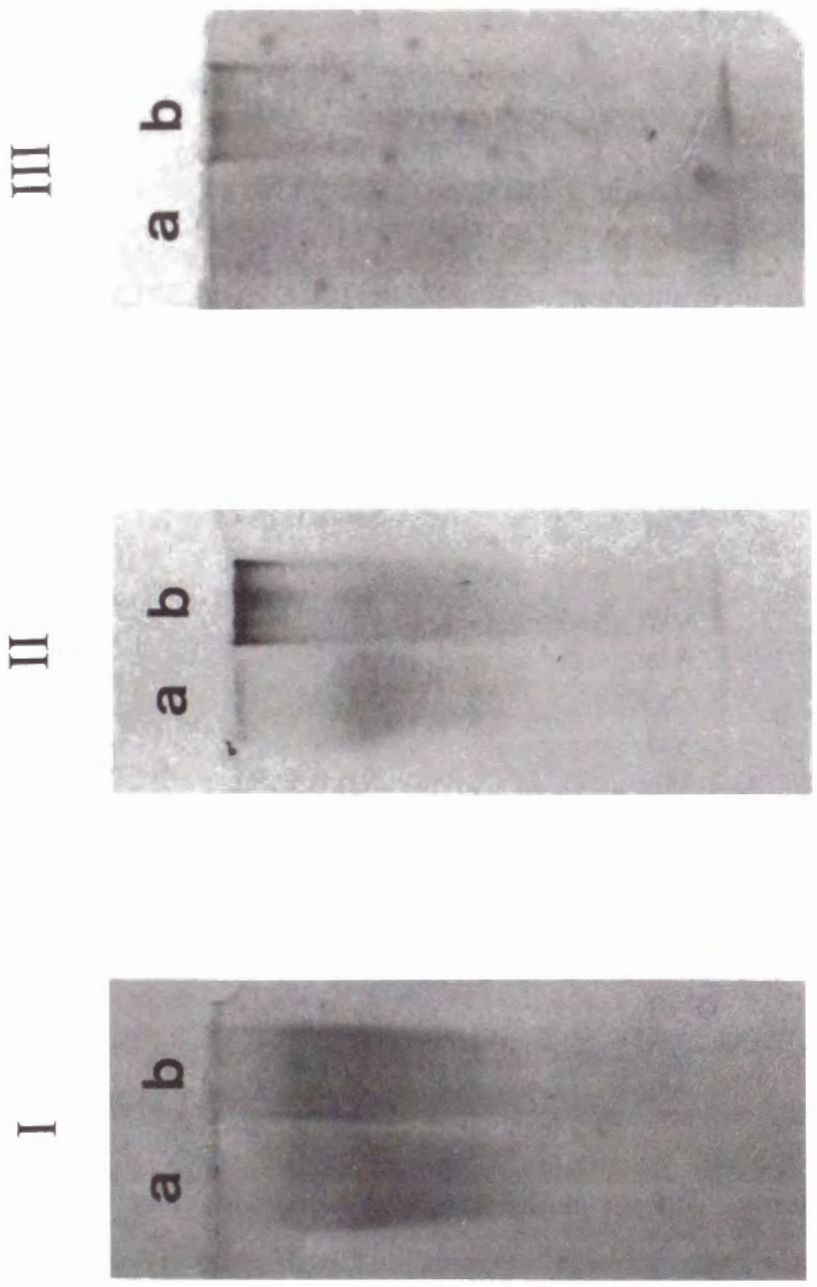

Fig. 5.8 Electrophretic patterns of salt-soluble (a) and water-soluble (b) arabinogalactan protein fom No.4.
Stained with β-galactosyl Yariv antigen (I), PAS reagent (II) and Commassi Brilliant Blue (III).

moiety. Water-soluble AGP was a kind of proteoglycan, but salt-soluble AGP in plasma membrane has a nature of glycoprotein. Since the result of the present chemical analysis did not agreed with that of the AGP in plasma membrane, the salt-soluble AGP is suggested to be a new type AGP, although its function is not clarified yet. Based on the result that the present salt-soluble AGP was extractable with aq. calcium chloride solution only after extractions with water, aq. CDTA and DMSO, the salt-soluble AGP was suggested to be present as in a highly sealed state in bamboo cell wall. Taking the suggestion of Yasufuku *et al.* (1994) that AGP was transformed into water-insoluble form during hardening of cabbage leaves into consideration, a modification of its protein moiety is suggested to occur in that transformation.

5.4 Summary

Water-soluble AGPs were isolated from 6 different portions of the same culm of immature Mosochiku. AGP was contained throughout the culm, but its content decreased with maturation. The water-soluble AGPs contain about 10 % of protein. Their carbohydrate moiety had common (1,3 : 1,6)-galactan portion with a few arabinofuranose residues as side chains. As their common protein constituents, hydoxyproline was occupied more than 40 %. These chemical properties indicate that the water-soluble AGP is closely related to the immaturity.

In addition, the author succeeded to isolate a new AGP having a glycoproteinic nature by further extraction with aq. calcium chloride solution. Its chemical properties differed from those of the water-soluble AGP in high protein content and low arabinose content. Because of high resistance for extraction, this salt-soluble AGP was suggested to be present as in a highly sealed state in bamboo cell wall.

Conclusion

Elongation growth mechanism of Mosochiku was investigated in respect of chemical changes in its cell wall constituents by using an immature culm of Mosochiku as the sample.

In Chapter 1, the immature culm of bamboo having 5 to 6 m in height was found to be appropriate to investigate the growth of bamboo. When chemical composition change was surveyed within a culm of immature Mosochiku having 6 m in height, accumulation of cellulose, hemicellulose, lignin and phenolic acids was found to proceed with lowering the position of the culm. The contents of protein, starch and ash were, however, higher in the higher portions. About 90 % of the phenolic acids were composed of *trans*-*p*-coumaric acid and *trans*-ferulic acid whose distribution within a culm was different in that *p*-coumaric acid increased with lowering the position in relation to lignin content, while a reverse relationship was observed in the ferulic acid. CP/MAS solid state ^{13}C-NMR was found to be useful to analyze the distribution of L-tyrosine.

In Chapter 2, internodal growth of the longest (17th) internode of a 6 m-high immature Mosochiku was characterized by analyzing chemical composition, X-ray diffraction and CP/MAS solid state ^{13}C-NMR. The growth of bamboo was concluded to occur upward from the secondary meristem localized at 1.06 to 2.13 cm above the knot. CP/MAS solid state ^{13}C-NMR was proved to be useful to determine the position of the secondary meristem.

In Chapter 3, variation of the distribution of esterified ferulic and *p*-coumaric acids during whole growth was found to be similar to that observed during internodal growth of Mosochiku by using UV and fluorescence microscopic techniques. The results indicate that esterified ferulic acid was synthesized at the early stage of growth and accumulated earlier than *p*-coumaric acid and lignin. The esterified

phenolic acids and lignin were synthesized from inner to outer layers of vascular bundle.

In Chapter 4, native arabinoxylan esterified with phenolic acids (ferulic and *p*-coumaric acids) was demonstrated to be present in DMSO-soluble polysaccharides from six different portions of an 6 m-high immature culm of Mosochiku. The contents of arabinoxylan and ferulic acid increased with lowering the position of the culm. The position of feruloylation was further demonstrated to be C-5 of arabinofuranose pendants by enzymatic hydrolysis and isolation of a known feruloylated arabinoxylobiose, *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose. Degree of feruloylation was estimated to increase with maturation from the top (one ferulic acid per 28 xylose residues) to the bottom (one ferulic acid per 4 xylose residues) of the culm.

In Chapter 5, water-soluble and hydroxyproline rich arabinogalactan protein (AGP) was isolated from 6 different portions of a 6 m-high immature culm of Mosochiku and found that its content was closely related to the immaturity. Isolated AGP, however, had a similar molecular weight of about 50 kDa and a common (1,3 : 1,6)-galactan portion with a few arabinofunanose side chains. In addition, a new type AGP having a glycoproteinic nature was found to be present in a highly sealed state in bamboo cell wall. Although both AGP had similar molecular weight, chemical composition of this AGP was different from that of the water-soluble AGP in low arabinose content.

Summarizing the results, the author demonstrated the usefulness of an immature culm for the investigation of elongation growth mechanism of Mosochiku and suggest the participation of feruloyl arabinoxylan and AGP in its growth.

References

Azuma, J., Nomura, T. and Koshijima, T. (1985) *Agric. Biol. Chem.*, **49**, 2661-2669.

Cartier, N., Chambat, G. and Joseleau, J.-P. (1987) *Carbohydr. Res.*, **168**, 275-283.

Clarke, A. E., Knox, R. B. and Jermyn, M. A. (1975) *J. Cell Sci.*, **19**, 157-167.

Clarke, A. E., Gleeson, P. A., Jemyn, M. A. and Knox, R. B. (1978) *Aust. J. Plant Physiol.*, **5**, 707-722.

Clarke, A. E., Anderson, R. L. and Stone, B. A. (1979) *Phytochmistry*, **18**, 521-40.

Dabrowski, K. J. and Sosulski, F. W. (1984) *J. Agric. Food Chem.*, **32**, 123-127.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.*, 28, 350-356.

Fairbanks, G., Steck, T. L. and Wallach D. F. H. (1971) *Biochemistry*, **10**, 2606-2617

Filisetti-Cozzi, T. M. C. C. and Carpita, N. C. (1991) *Anal. Biochem.*, **197**, 157-162.

Fincher, G. B. (1976) *J. Inst. Brew.*, **82**, 347-349.

Fincher, G. B., Stone, B. A. and Clarke, A. E. (1983) *Ann. Rev. Plant Physiol.*, **34**, 47-70.

Fry, S. C. (1979) *Planta*, **146**, 343-351.

Fry, S. C. (1983) *Planta*, **157**, 111-123.

Fujii, Y., Azuma, J., Takabe, K. and Okamura, K. (1991) *Bull. Kyoto Univ. For.*, **63**, 272-282.

Fujii, Y., Azuma, J. and Okamura, K. (1992) In : Plant cell walls as biopolymers with physiological functions. (Masuda, Y. ed.) pp.419-421, Yamada Science Foundation, Japan.

Fujii, Y., Azuma, J., Marchessault, R. H., Morin, F. G., Aibara, S. and Okamura, K. (1993a) *Holzforschung*, **47**, 109-115.

Fujii, Y., Azuma, J. and Okamura, K. (1993b) *Bamboo J.*, **11**, 41-53.

Fulcher, R. G., O' Brien, T. P. and Lee, J. W. (1972) *Aust. J. Bio. Sci.*, **25**, 23-34.

Fyfe, C. A. (1983) In : Solid State NMR For Chemists. p.475. C.F.C. Press, Guelph, Ontario, Canada.

Geissman, T. and Neukom, H. (1973) *Lebensm.-Wiss. u. Technol.*, **6**, 59-62.

Grosser, D. and Liese, W. (1971) *Wood Sci, Technol.* **5**, 290-312.

Gubler, F., Ashford, A. E., Bacic. A., Blakeney, A. B. and Stone, B. A. (1985) *Aust. J. Plant Physiol.*, **12**, 307-317.

Hägglund, E., Lindberg, B. and McPherson, J. (1956) *Acta Chem. Scand.*, **10**, 1160-1164.

Hakomori, S. (1964) *Biochem. J.*, **55**, 205-207.

Harborne, J. B. and Corner, J. J. (1961) *Biochem. J.*, **81**, 242-250.

Harris, P. J. and Hartley, R. D. (1976) *Nature*, **259**, 508-510.

Harris, P. J. and Hartley, R. D. (1980) *Biochem. Syst. Ecol.*, **8**, 153-160.

Hartley, R. D. and Harris, P. J. (1981) *Biochem. Syst. Ecol.*, **9**, 189-203.

He, L. and Terashima, N. (1989) *Mokuzai Gakkaishi*, **35**, 123-129.

Higuchi, T., Kawamura, I and Ishikawa, H. (1953) *J. Jan. For. Soc.*, **35**, 258-263.

Higuchi, T. (1966) *Agric. Biol. Chem.*, **30**, 667-673.

Higuchi, T., Kimura, N. and Kawamura, I. (1966) *Mokuzai Gakkaishi*, **12**, 173-178.

Higuchi, T., Ito, Y. and Kawamura, I. (1967a) *Phytochemistry*, **6**, 875-881.

Higuchi, T., Shimada, M. and Ohashi, H. (1967b) *Agric. Biol. Chem.*, **31**, 1459-1465.

Higuchi, T. and Shimada, M. (1969) *Phytochemistry*, **8**, 1183-1192.

Higuchi, T. (1987) *Bamboo J.*, **4**, 132-145.

Humphreys, F. R. and Kelly, J. (1961) *Anal. Chem. Acta*, **24**, 66-70.

Idei, T. (1981) *Bull. Utunomiya Univ. For.*, **17**, 59-73.

Ishii, T. and Hiroi, T. (1990a) *Carbohydr. Res.*, **196**, 175-183.

Ishii, T. and Hiroi, T. (1990b) *Carbohydrate Res.*, **206**, 297-310.

Ishii, T. Hiroi,T. and Thomas, J. R. (1990c) *Phytochemistry*, **29**, 1999-2003.

Ishii, T.(1991) *Carbohydr. Res.*, **219**, 15-22.

Itoh, T. (1990) *Holzforschung*, **44**, 191-200.

Johansson, A., Lindberg, B. and Theander, O. (1954) *Svensk Papperstidning*, **31**, 41-432.

Kamisaka, S., Takeda, S., Takahashi, K. and Shibata, K. (1990) *Physiol. Plant.*, **78**, 1-7.

Karacsonyi, S., Kovacik, V., Alfoldi, J. and Kumackova, M. (1984) *Carbohydr. Res.*, **134**, 265-274.

Kato, A., Azuma, J. and Koshijima ,T. (1983) *Chem. Letters*, 137-140.

Kato, Y., Shiozawa, R., Takeda, S. Ito, S. and Matsuda, K. (1982) *Carbohydr. Res.*, **109**, 233-248.

Kikuchi, S., Ohinata, A., Tsumuraya, Y., Hashimoto, Y., Kaneko, Y. and Matsushima, H. (1993) *Planta*, **190**, 525-535

Kolodziejski, W., Frue, J. S. Frye and Maciel, G. E. (1982) *Anal. Chem.*, **54**, 1419-1424.

Komalavilas, P., Zhu, J.-K. and Nothnagel, E. A. (1991) *J. Biol. Chem.*, **266**, 15956-15965.

Koshimizu, K. and Mitsui, T. (1955) *Nougei Kagaku Kaishi*, **30,** 63-65.

Kozukue, E. and Kozukue, N. (1981) *J. Food Sci.*, **46**, 751-755.

Kozukue, E., Kozukue, N. and Kurosaki, T. (1983) *J. Food Sci.*, **48**, 935-938.

Krygier, K. Sosulski, F. and Hogge, L. (1982) *J. Agric. Food Chem.*, **30**, 330-334.

Kuhn, R. and Terischmann H. (1963) *Ber.* **96**, 284-287.

Liese, W. (1987) *Wood Sci. Technol.*, **21**, 189-201.

Lindberg, B. (1972) *Methods Enzymol.*, **28**, 178-195.

Maciel, G. B., Haw, J. F., Smith, D. H., Gabrielsen B. C. and Hatfield G. R. (1985) *J. Agric. Food Chem.*, **331**

Maekawa, E. and Kitao, K. (1973) *Agric. Biol. Chem.*, **37**, 2445-2447.

Maekawa, E. and Kitao, K. (1974) *Agric. Biol. Chem.*, **38**, 227-229.

Maekawa, E (1975a) *Agric. Biol. Chem.*, **39**, 2281-2289.

Maekawa, E (1975b) *Agric. Biol. Chem.*, **39**, 2291-2296.

Majima, S., Fujita, M. and Saiki, H. (1991) *Bull. Kyoto Univ. For.*, **63**, 236-245.

Marchessault, R. H., Taylor, M. G., Hamer, G. and Deslandes, Y. (1985) In : Papermaking raw materials - Their interaction with the production process and their effect on paper properties, vol.1. (V. Punton, ed.) , pp.37-57. Mechanical Engineering Publications Ltd., London.

Mascara, T. and Fincher, G. B. (1982) *Aust. J. Plant Physio*l, **8**, 31-45.

Matsuzaki, K. Moriya, M and Sobue, H. (1960) *Kogyo Kagaku Zasshi*, **63**, 638-639.

Matsuzaki, K. Moriya, M and Sobue, H. (1962) *Kogyo Kagaku Zasshi*, **65**, 987.

McClure, F. A. (1966) In : The Bamboos -A Fresh Perspective. p.45 and pp122-126. Harvard University Press, Cambridge, Massachusetts.

Mochizuki, T. and Kurosaki, T. (1988) *Bamboo J.*, **6**, 47-55.

Mori, M., Yamazaki, T. and Shimizu, T. (1978) *Sagami Joshi Daigaku Zashi*, **42**, 19-24.

Muroi, H. (1992) *Take*, **46**, 2-5.

Musha, Y. and Goring, D. A. I. (1975) *Wood Sci. Technol.*, **9**, 45-58.

Nakamura, Y. and Higuchi, T. (1976) *Holzforschung*, **30**, 187-191.

Nomura, T. and Yamada, T. (1974a) *Wood Res.*, **56**, 21-27.

Nomura, T. and Yamada, T. (1974b) *Wood Res.*, **57**, 23-30.

Ochterlony, O. (1958) *Progr. Allergy*, **5**, 1-9.

Ornstein, L. and Davis, B. (1964) *Ann. N.Y. Acad. Sci.*, **121**, 321-349.

Pennell, R. I., Knox, J. P., Scofield, G. N., Selvendran, R. R. and Roberts, K. (1989) *J. Cell Biol.*, **108**, 1967-1977.

Pennell, R. I. (1992) In : Perspectives in plant cell recognition, Society for experimental biology seminar series vol. 48. (Callow, J. A. and Green, J. R. ed.), pp105-129. Cambridge University Press, Cambridge.

Pollard, P. C. and Fincher, G. B. (1981) *Aust. J. Plant Physiol.*, **8**, 121-132.

Sachs, I. B., Clark, I. T. and Pew, J. C. (1963) *J. Polymer Sci. C*, **2**, 203-212.

Saeman, J. F., Moore, W. E., Mitchell, R. L. and Millett, A. M. (1954) *Tappi*, **37**, 336-343.

Sanford, P. A. and Connrad, H. E. (1966) *Biochemistry*, **5**, 1508-1516.

Saulnier, L. and Brillouet, J.-M. (1988) *Carbohydr. Res.*, **182**, 63-78.

Schaefer, J., Stejskal, E. O. and Buchdahl, R. (1977) *Macromolecules*, **10**, 385-405.

Schopfer, P. (1990) *Planta*, **183**, 139-142.

Segal, L., Creely, J. J., Martin Jr., A. E. and Conrad, C. M. (1959) *Textile Res. J.*, **29**, 786-794.

Shibamoto, T., Shoji, R. and Kubota, S. (1954a) *Bull. Tokyo Univ. For.*, **47**, 203-207.

Shibamoto, T., Shoji, R. and Tagawa, K. (1954b) *Bull. Tokyo Univ. For.*, **47**, 209-219.

Shimada, M., Yamazaki, T. and Higuchi, T. (1970) *Phytochemistry*, **9**, 1-4.

Shimada, M., Fukuzaka, T. and Higuchi, T.(1971) *Tappi*, **54**, 72-78

Taylor, M. G., Deslandes, Y., Bluhm, T., Marchessault, R. H., Vincendon, M. and Saint-germain, J. (1983) *Tappi J.*, **66**, 92-94.

Taniguchi, E. (1956) *Mokuzai Gakkaishi*, **2**, 152-157.

Tsumuraya, Y., Ogura, K., Hashimoto, Y., Mukoyama, H. and Yamamoto, S. (1988) *Plant Physiol.*, **86**, 155-160.

Ueda, K. (1960) *Bull. Kyoto Univ. For.*, **30**

Uprichard, J. M. (1965) *Appita*, **19**, 36-39.

Yariv, J., Rapport, M. M. and Graf, L. (1962) *Biochem. J.*, **85**, 383-388.

Yariv, J., Lis, H. and Katchalaski, E. (1967) *Biochem. J.*, **105**, 1C-2C

Yasufuku, H., Azuma, J., Kido, S. and Koshijima, T. (1985) *Agric. Biol. Chem.* **49**, 3429-3435.

Yasufuku, H., Kido, S., Azuma, J. and Okamura, K. (1987) *Nippon Nogeikagaku Kaishi*, **61**, 809-815.

Yasufuku, H., Kido, S., Azuma, J. and Okamura, K. (1994) *Biosci. Biotech. Biochem.*, **58**, 225-229.

Yoshinaga, A., Fujita, M. and Saiki, H. (1989) *Bull. Kyoto Univ. For.* **61**, 276-284.

Yoshizawa, N. and Idei., T. (1980) *Bull. Utunomiya Univ. For.*, **16**, 55-64.

Yoshizawa, N., Satoh, I., Yokota, S. and Idei, T. (1991) *Holzforshung*, **45**, 169-174.

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